THE BIOCHEMICAL JOURNAL

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Edited for

THE BIOCHEMICAL SOCIETY

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Obituary Notice

ALEXANDER THOMAS CAMERON, 1882–1947

Alexander Thomas Cameron, Professor of Biochemistry in the University of Manitoba, and an original member of the Biochemical Society, died at his residence in Winnipeg on 25 September 1947.

Born in London in 1882, of Scottish parentage, he received his early education in Swindon, proceeding thence to the University of Edinburgh from which he graduated M.A. in 1904 and B.Sc. in 1906. In the latter year he was awarded an 1851 Exhibition Scholarship, and, his interests lying in the field of physical chemistry, for the next two years he studied radiochemistry at University College, London, under Sir William Ramsay, followed by a year at the Technical High School, Karlsruhe, under Fritz Haber. In 1909 he was appointed Lecturer in Physiology in the University of Manitoba, an event which proved to be the turning point in his career.

With this appointment his attention was diverted to the ill-defined field of physiological chemistry in which he speedily found problems with which his training and experience in the methods of physical chemistry enabled him to cope successfully. There, too, under the dynamic influence of Swale Vincent, he was introduced to the study of what were then known as the 'ductless glands', and commencing with an investigation of the distribution of iodine in plant and animal tissues, he laid the foundations of his reputation as an endocrinologist.

Except for a summer semester spent in research under Albrecht Kossel at the University of Heidelberg, and three years during the first World War when, as Captain, R.A.M.C., he acted as chemist officer for water purification with the British Expeditionary Force in France, his subsequent career was intimately linked with the University of Manitoba. The growing importance of physiological chemistry inevitably led to a separation from the parent department of physiology, and this was recognized by the institution in 1923 of a separate Chair of Biochemistry in the Faculty of Medicine, and the appointment of Cameron as Professor.

An indefatigable and painstaking worker, Cameron was author or joint author of over 100 published papers. His first paper, published while still a student at Edinburgh, appeared in the *Proceedings of the Royal Society of Edinburgh* in 1905. It dealt with the crystallization of potassium hydrogen succinate, and was followed by a series dealing with physicochemical concepts, including the effect of heat and cold on cold-blooded animals. His publications dealing with the biochemistry of iodine (for which he was awarded the degree of D.Sc. by the University of Edinburgh in 1925) were the forerunners of a long series on the effect of thyroid and thyroxine feeding on the growth rate and organ hypertrophy of the white rat. Another series of papers dealt with the biochemistry of calcium, hypoparathyroidism, and tetany, while other publications included the results of studies on creatine and creatinuria, pernicious anaemia, the production of tar carcinoma in mice, etc. The last of the long list was a monograph, the final proofs of which were submitted for his approval just prior to his death. It was entitled *The Taste Sense and the Relative Sweetness of Sugars and other Sweet Substances*, and embodied the results of some three years of experimental work in that little-known field.

He was, however, best known for his text books. Never a very fluent lecturer, he recognized his limitations in that respect, and carefully prepared his lectures in biochemistry by writing them out in full. Since at that time there was no concise and authoritative text on Biochemistry, on the urging of his students and friends he submitted the manuscripts to a well known firm of publishers. Thus was born Cameron's *Textbook of Biochemistry*, the success of which is borne out by the fact that since its initial appearance in 1928 it has gone through six editions, as well as a Chinese and two Spanish editions. It was followed two years later by *Practical Biochemistry* (Cameron & White), now in its fifth edition. In 1933 two more books appeared, the result of his outstanding ability to present in clear and concise form a comprehensive and critical appreciation of a subject. These were *Biochemistry of Medicine* (Cameron & Gilmour) and *Recent Advances in Endocrinology*, of which the latter is now in its sixth edition and has been translated into Italian and Roumanian.

Fully occupied though he was with his teaching, writing, and research work, Cameron still had time for the public duties which fell to him as Chairman of the Fisheries Research Board of Canada, a position he held with conspicuous success for thirteen years. In this capacity he was chosen as one of the Canadian delegates to the Empire Scientific Congress held in London and Oxford in the summer of

1946. While attending these meetings he received official notification that his services had been recognized by the award of the C.M.G.

Cameron was a Fellow of the Royal Society of Canada, a Fellow of the Royal Institute of Chemistry, a Past President of the Canadian Institute of Chemistry (now the Chemical Institute of Canada), and a member of various chemical, biochemical, and medical societies. One of his life-long interests was the Scientific Club of Winnipeg, of which he was one of the early members, for twelve years its Secretary, and to whose Scientific Proceedings he made 46 contributions.

Although of a very reserved nature, Cameron made friends in all parts of the Dominion, and they, together with many others to whom he was only known by name, mourn the passing of a scientist and scholar.

F. D. WHITE
J. B. COLLIP

A Note on the Estimation of Vitamin B_1 in Urine

By E. H. MAWSON and S. Y. THOMPSON
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(*Received* 6 *October* 1947)

The accurate measurement of the vitamin B_1 content of urine by the thiochrome method is made difficult by the interference of non-specific fluorescent substances. Adsorption on the zeolite Decalso (The Permutit Co. Ltd.) as described by Hennessy & Cereceedo (1939) removes some of these, but not nicotinamide methochloride which occurs in the urine of man and some other animals including the rat. Treatment with alkali, ferricyanide and *iso*butanol in the thiochrome procedure converts nicotinamide methochloride to a violet-fluorescing compound (F_3) (Coulson, 1944), which is indistinguishable fluorimetrically from thiochrome. When ferricyanide is omitted, another substance (F_2) with a bluish-white fluorescence is formed from nicotinamide methochloride (Najjar & Holt, 1941). This makes the so-called 'NaOH blank' unsatisfactory. Several ways of overcoming this difficulty have been proposed (Mason & Williams, 1942; Najjar & Ketron, 1944; Coulson, 1944; Mickelsen, Condiff & Keys, 1945), but for reasons which will be given subsequently we believe that none of them is entirely satisfactory.

During work on the effect of sulphonamides on the excretion of vitamin B_1 by rats, we developed a procedure for avoiding interference by nicotinamide methochloride which, in our opinion, is an improvement on those at present in use. It depends on the fact that the blue fluorescence of thiochrome disappears on addition of acid to the *iso*butanol extract, whereas the fluorescence of F_3 is unaffected. By measuring the fluorescence before and after the addition of acid, a measure of the fluorescence due to thiochrome is obtained.

METHOD

A sample of urine (2–20 ml.), containing if possible 2–3 μg. vitamin B_1, is adjusted to pH 4·5 with glacial acetic acid, and diluted to 50 ml. with glass-distilled water. One 25 ml. portion is poured on to a Decalso column 5 cm. long and 0·6 cm. in diameter; 1·3 μg. vitamin B_1 is added to the other 25 ml. portion which is poured on to another column. The shape and size of the adsorption tube and the activation of the zeolite are as described by Hennessy (1941). The columns are washed three times with 10 ml. water, and the vitamin B_1 is then eluted with successive portions of 3, 3 and 5 ml. 25% (w/v) KCl in 0·1 N-HCl. The volume of the eluate is adjusted to 11·0 ml.

For oxidation to thiochrome, 40% NaOH (1 ml.), freshly prepared 1% $K_3Fe(CN)_6$ (0·1 ml.) and redistilled *iso*butanol (15 ml.) are added to a 5 ml. portion of the eluate in a glass-stoppered 2 oz. bottle. The contents of the bottle are well mixed after each addition and allowed to stand for 1 hr. If measurement of the fluorescence of F_2 is desired, a second bottle may be prepared in the same way except that ferricyanide is omitted, but this is not part of our routine procedure. Otherwise, the second 5 ml. portion of the eluate may be used for a duplicate determination of vitamin B_1.

Measurement of the fluorescence in a Cohen-type instrument (Henry, Houston, Kon & Osborne, 1939) is made with a 10 ml. portion of the *iso*butanol layer. While this is still in the test tube of the fluorimeter, seven drops of a mixture of three parts methanol and four parts N-HCl are added, the contents mixed, and the fluorescence measured again. The difference between the two readings is a measure of the fluorescence of thiochrome. This procedure makes the 'NaOH blank' unnecessary for the measurement of vitamin B_1, but useful as an indication of the amount of nicotinamide methochloride present.

It should be mentioned that this technique cannot be used with extracts containing methanol, which markedly increases the solubility of alkali in the *iso*butanol phase, as addition of sufficient HCl to neutralize this alkali leads to turbidity.

RESULTS

The results of an experiment, in which pure substances were used (Table 1), show that the fluorescence of thiochrome, as observed visually and as measured fluorimetrically, disappears after treatment with acid whereas that of F_3 does not. The galvanometer deflexion due to thiochrome, obtained by difference, is almost the same for vitamin B_1 alone as in the presence of nicotinamide methochloride. Use of the 'NaOH blank' (F_2) would give a negative result for vitamin B_1 in the presence of nicotinamide methochloride. In our fluorimeter the fluorescence of F_2 appears greenish instead of bluish white.

Typical results obtained with normal human urine, and with urine of normal and vitamin B_1-deficient rats, are given in Table 2. Two assumptions are unavoidable in the application of our method, as of many other methods for the estimation of vitamin B_1 in urine. One is that vitamin B_1 is adsorbed and eluted to the same extent from the zeolite columns, through which the unknown with and without added vitamin B_1 had been passed; the other is that thiochrome is extracted to the same degree from eluates with and without added vitamin B_1. On the other hand, the measurement of the blank with the same *iso*butanol extract used for measurement of the unknown obviates two other possible sources of error: (*a*) that due to differences in the extent to which nicotinamide methochloride is adsorbed on, and eluted from, different zeolite columns, and (*b*) that due to differences in the extent to which F_3 and other fluorescent substances (not thiochrome) are ex-

Table 1. *The effect of* HCl *treatment on the fluorescence of thiochrome and of* F_3

	Substance and quantity taken for zeolite adsorption					
	Vitamin B_1 (2·57 μg.)		Nicotinamide methochloride (250 μg.)		Vitamin B_1 (2·57 μg.) + nicotinamide methochloride (250 μg.)	
Treatment	Galvanometer deflexion*	Colour of fluorescence	Galvanometer deflexion*	Colour of fluorescence	Galvanometer deflexion*	Colour of fluorescence
(1) NaOH, then *iso*butanol extraction	24	Not visible	228	Greenish	218	Greenish
(2) NaOH ferricyanide, then *iso*butanol extraction	138	Blue	102	Violet	207	Bluish violet
(3) *iso*Butanol extract (2) treated with HCl	35	Not visible	103	Violet	102	Violet
Deflexion due to thiochrome ((2) – (3))	103	—	−1	—	105	—

* Linear scale.

Table 2. *The determination of vitamin* B_1 *in rat and human urine by the thiochrome method involving the use of* HCl

(Values are given as galvanometer deflexions, linear scale.)

	Rat urine				Human urine	
	Normal diet		Vitamin B_1 deficient diet			
Treatment	Without added vitamin B_1*	With 1·3 μg. added vitamin B_1*	Without added vitamin B_1†	With 1·3 μg. added vitamin B_1†	Without added vitamin B_1‡	With 1·3 μg. added vitamin B_1‡
(1) NaOH, then *iso*butanol extraction	142	140	320	360	364	374
(2) NaOH and ferricyanide, then *iso*butanol extraction	236	300	79	158	161	233
(3) *iso*Butanol extract (2) treated with HCl	58	56	80	89	76	77
Deflexion due to thiochrome ((2) – (3))	178	244	−1	69	85	156
Calculated vitamin B_1 content of urine	2·7 μg./ml.		0·0 μg./ml.		0·16 μg./ml.	

* 1 ml. urine taken. † 4 ml. urine taken. ‡ 10 ml. urine taken.

Table 3. *Urinary excretion of vitamin B_1 by rats with different intakes of vitamin B_1*

Treatment	No. of observations	Urinary excretion (μg./rat/day) Mean	Range
Diet deficient in vitamin B_1	7	0	0–0
Diet deficient in vitamin B_1 + 20 μg. vitamin B_1 daily	11	0·36	0·1–0·6
Diet deficient in vitamin B_1 + 40 μg. vitamin B_1 daily	3	1·89	1·3–3·0

Table 4. *A comparison of the determination of vitamin B_1 in pure solutions in the presence and absence of nicotinamide methochloride by the method of Mickelsen et al.* (1945) *and by our method*

(Values are given as galvanometer deflexions, linear scale.)

Treatment	Substance and quantity taken for zeolite adsorption: Vitamin B_1 (1·3 μg.)	Nicotinamide methochloride (250 μg.)	Nicotinamide methochloride (250 μg.) + vitamin B_1 (1·3 μg.)
(1) NaOH, then *iso*butanol extraction at pH 12	34	270	293
(1*a*) NaOH, then *iso*butanol extraction at pH 8–9·5	40	37	39
(2) NaOH and ferricyanide, then *iso*butanol extraction at pH 12	98	103	180
(2*a*) NaOH and ferricyanide, then *iso*butanol extraction at pH 8–9·5	97	108	175
(3) *iso*Butanol extract (2) treated with HCl	37	103	116
Deflexion attributed to vitamin B_1:			
Method of Mickelsen *et al.*: ((2*a*) − (1*a*))	57	71	136
Our method: ((2) − (3))	61	0	64

Table 5. *A comparison of measurement of vitamin B_1 in rat and human urine, by the method of Mickelsen et al.* (1945) *and by our method*

(Values given as galvanometer deflexions, linear scale.)

Treatment	Urine* of rats on vitamin B_1 deficient diet: Without added vitamin B_1	With added vitamin B_1 (1·3 μg.)	Normal human urine†: Without added vitamin B_1	With added vitamin B_1 (2·6 μg.)
(1) NaOH, then *iso*butanol extraction at pH 12	400	400	142	150
(1*a*) NaOH, then *iso*butanol extraction at pH 8·0–9·5	39	40	27	28
(2) NaOH and ferricyanide, then *iso*butanol extraction at pH 12	148	225	68	158
(2*a*) NaOH and ferricyanide, then *iso*butanol extraction at pH 8·0–9·5	148	226	63	147
(3) *iso*Butanol extract (2) treated with HCl	148	150	47	50
Method of Mickelsen *et al.* ((2*a*) − (1*a*)):				
Deflexion attributed to vitamin B_1	109	185	36	119
Calculated vitamin B_1 content of urine	0·45 μg./ml.		0·11 μg./ml.	
Our method ((2) − (3)):				
Deflexion attributed to vitamin B_1	0	75	21	108
Calculated vitamin B_1 content of urine	0·0 μg./ml.		0·06 μg./ml.	

* 4 ml. urine taken.

† 10 ml. urine taken.

tracted by *iso*butanol in separate samples of the extract. We have frequently observed that the blank obtained for urine with added vitamin B_1 is slightly higher than that for urine alone, which may be due to a greater elution of nicotinamide methochloride from the zeolite column in the presence of additional vitamin B_1.

Results similar to those in Table 2 have been obtained with human urine on many occasions, but our experience has been primarily with rat urine. Typical results (Table 3) show that the excretion of vitamin B_1 by rats, as determined by our method, is closely related to the vitamin B_1 content of the diet.

DISCUSSION

We believe that our procedure is an improvement on those of other workers for the following reasons. We are in agreement with Najjar & Ketron (1944) that nicotinamide methochloride is partly destroyed by sodium sulphite and that the procedure suggested by Mason & Williams (1942) gives too high results. Najjar & Ketron (1944) and Coulson (1944) allow for the interference of nicotinamide methochloride by assuming that the galvanometer deflexion due to F_2 in the NaOH blank bears a fixed relation to that due to F_3 in the unknown. No such assumption is necessary in our method as we are able to measure F_3 directly.

In the method of Mickelsen *et al.* (1945) the *iso*butanol extraction is carried out at a pH 8·0–9·5, at which F_2 in the NaOH blank does not fluoresce, but the fluorescence of thiochrome in the unknown is unimpaired. Table 4 confirms this but shows that this treatment does not affect F_3, so that in the presence of nicotinamide methochloride both thiochrome and F_3 contribute to the fluorescence. Table 5 shows that for this reason the method of Mickelsen *et al.* (1945) gives too high results when applied to rat and human urine; e.g. the urine of vitamin B_1-deficient rats was found by this method to contain 0·45 μg. vitamin B_1/100 ml., whereas none was found by our method. For the sample of human urine the discrepancy was less since less nicotinamide was present.

SUMMARY

A modification of the thiochrome method for the fluorimetric determination of vitamin B_1 in urine in the presence of nicotinamide methochloride is described. Since treatment of the *iso*butanol extract with HCl removes the thiochrome fluorescence, the difference between readings taken before and after this treatment is a measure of the vitamin B_1 content of the urine.

We wish to thank Dr A. C. Bottomley for preparing the nicotinamide methochloride.

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Aggregate Formation in Soil

1. INFLUENCE OF SOME BACTERIAL POLYSACCHARIDES ON THE BINDING OF SOIL PARTICLES

By M. J. GEOGHEGAN and R. C. BRIAN
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(*Received* 10 *October* 1947)

For maximum crop production it is essential that the soil should have a good crumb structure and that the crumbs should be stable and resistant to the dispersing effects of rain, frost, etc. Aggregate formation facilitates cultivation, drainage and aeration, increases the moisture-holding capacity of soil and reduces erosion. It also maintains sufficient cohesion in the soil to give anchorage to plants, and yet sufficient incoherence to facilitate root penetration and emergence of seedlings. Large additions of organic matter and the growth of grassland vegetation are credited with having an ameliorative effect on the physical state of the soil, but there is considerable diversity of opinion as to the exact processes involved in the formation of water-stable aggregates.

Stable structure formation under natural conditions is possibly a gradual process influenced by many factors. It is reasonable to expect that physical, chemical and biological agencies are involved. The biological agencies include plants, animals and micro-organisms; and of these the micro-organisms are of great importance in pro-

ducing cementing or stabilizing substances, and filamentous forms like fungi, actinomyces and some algae may have a mechanical binding effect.

Geoghegan & Brian (1946) reported that bacterial polysaccharides of the levan and dextran types have a marked aggregating effect on soil particles. Levans in which fructofuranose units are joined by 2:6 linkages are formed by a wide variety of aerobic organisms including plant pathogens. Dextrans, polyglucoses in which the units are joined by 1:6 glucosidic linkages, are formed by various species of *Leuconostoc* bacteria and possibly other organisms.

As a preliminary to finding how these bacterial products aggregate soil particles, exceptionally pure samples of levans and dextrans have been prepared and examined in some detail.

EXPERIMENTAL

Bacterial strains used. An organism, isolated from garden soil and identified as a strain of *Bacillus subtilis*, was used for levan synthesis. Cultures of *Leuconostoc dextranicum* (N.C.T.C. Cat. no. 3354) and *Leuconostoc mesenteroides* (N.C.T.C. Cat. no. 3351) were obtained from the National Collection of Type Cultures and used for dextran production.

Media used. Media having the following composition were employed:

A		B	
KH_2PO_4	1·5 g.	K_2HPO_4	1·0 g.
$NaNO_3$	2·0 g.	$Na(NH_4)HPO_4.4H_2O$	4·9 g.
$MgSO_4.7H_2O$	0·5 g.	$MgSO_4.7H_2O$	0·5 g.
KCl	0·3 g.	KCl	0·5 g.
Distilled water	1000 ml.	$FeSO_4.7H_2O$	0·01 g.
		Bactopeptone	2·0 g.
		Distilled water	1000 ml.

Sucrose was added to (A) in amounts sufficient to give 2, 4, 6, 8 and 10% (w/v) concentrations of sucrose in the final medium and to (B) to give 2 and 10% (w/v). The media were adjusted to pH 7·0, dispensed in litre quantities in 1500 ml. Erlenmeyer flasks and sterilized by autoclaving under 15 lb./sq.in. pressure for 15 min. To avoid precipitation it was necessary to sterilize the $MgSO_4.7H_2O$ separately, and after cooling to add it to the rest of the cooled medium employing aseptic technique.

Production of levans. The media were inoculated with the washings of 48 hr.-old agar-slant cultures of the organism, and after 8–10 days' incubation at 27° large amounts of polysaccharides were formed. The gross impurities were removed by filtering the solutions through no. 1 Whatman filter paper and the liquids were concentrated *in vacuo*. Following centrifugation and dialysis, sufficient ethanol was added to give 70% (v/v) in the final solutions, and the precipitated polysaccharides were obtained as white products. These crude materials, resuspended in water, formed viscous opalescent solutions, which contained large amounts of bacterial cells and 'cell debris'. In case nucleoproteins were present the solutions were adjusted to pH 4·0 and centrifuged. The precipitate was discarded and the supernatant fluid dialyzed until it had a pH of 7·0. Finally, proteins and bacterial cells were removed by the method of Sevag (1934) and the purified levans were precipitated with ethanol, dried, and ground to fine powders.

Production of dextrans. The media were inoculated with 5 ml. of 4-day-old sucrose-broth cultures of the organisms and, except in the case of the product from *Leuconostoc dextranicum*, the method of isolation and purification of the dextrans was the same as for levans. The dextran produced by *L. dextranicum* was insoluble in water after ethanolic precipitation; hence in the isolation of this polysaccharide the first precipitation with ethanol was omitted.

Hydrolysis of levans. The purified levans were dissolved in excess 0·1 N-H_2SO_4 and heated under reflux for 30 min. The hydrolysates were cooled to 60° and neutralized with solid $BaCO_3$, rapidly cooled and the $BaSO_4$ removed by centrifugation. The clear solutions were then made up to the required volumes.

Hydrolysis of dextrans. The method of hydrolysis in this case was identical with that used for levan, except that 2 N-H_2SO_4 was used and heating was continued for 3 hr.

Estimations

(1) *Total reducing sugars.* According to the Shaffer-Somogyi method (Heinze & Murneek, 1940).

(2) *Fructose.* By the 'ferricyanide method' of Becker & Englis (1941). Methyl red was used as indicator instead of sodium diphenylaminesulphonate.

(3) *Total* N. By the micro-Kjeldahl method (Pregl, 1930).

(4) *Aggregating effect.* A soil, the structure of which in terms of water-stable crumbs is extremely poor, was air-dried and ground to pass through a 52-mesh B.S.S. sieve. The polysaccharides, in colloidal suspension, were applied so that different samples of soil contained from 0·05 to 0·75% polysaccharide. Sufficient moisture was used to bring the soil to the 'sticky point'. In this state it was well stirred with a glass rod, placed on a 3 mm. sieve and pressed through it.

The aggregates were dried at 50° and their stability was tested by the following technique: 25 g. of aggregates were placed in 600 ml. of water in a 1 l. cylinder. The cylinder was inverted once and then allowed to stand for 30 min. After this the cylinder was inverted 30 times, the operation taking about 1 min. The contents were then poured on to a 52-mesh B.S.S. sieve (8 in. diameter) standing in a shallow bowl. Extra water was added to bring the level above that of the soil on the sieve. The sieve was then moved up and down 30 times, the water allowed to drain out each time; the sieve was gently tilted each time. The sieve was then removed, the contents washed into a basin and the whole evaporated to dryness (105°). The weight of water-stable aggregates is expressed as a percentage of the air-dried soil. The control samples (moistened with water, granulated and dried) were completely unstable when they were wet-sieved. Apparently, wetting and drying or pressing of moist soil through a sieve, *per se*, is unable to produce water-stable aggregates.

The advantages of the above method are that there is very good agreement between replicates, results are reproducible and small differences in the aggregating abilities of the polysaccharides are easily measured.

Viscosity measurements. To avoid terminological confusion, so common in this field, we adopted throughout the symbols and nomenclature proposed by Cragg (1946).

The measurement of viscosities of levan and dextran solutions involved the determination of the rate of flow of the solutions in an Ostwald viscometer. All solutions were thoroughly centrifuged before being run into the viscometer. The flow time and density (pyknometer method) of levan and dextran solutions, varying in concentration from 1·5 to 0·125% (w/v), and weaker dilutions where necessary, were determined at 20±0·1°, and from the data the relative viscosity (η_r) was calculated.

If d_1 = density of solvent,
d_2 = density of polysaccharide solution,
t_1 = flow time of solvent,
t_2 = flow time of polysaccharide solution,

then $\eta_r = \frac{t_2 d_2}{t_1 d_1} = \frac{t_2 d_2}{t_1}$ when the solvent is water, and the specific viscosity $(\eta_{sp}) = \eta_r - 1$.

Hence the reduced viscosity (η_{sp}/c) may be calculated, and if this is plotted against the corresponding concentration c (in primary moles) the intrinsic viscosity

$$\lim_{c \to 0} \left(\frac{\eta_{sp}}{c} \right)$$

is obtained.

RESULTS

Bacillus subtilis was cultured in a number of different media in which the sucrose concentration and source of nitrogen were varied, and in every case there was marked opalescence and an increase in viscosity associated with the formation of a polysaccharide which was a levan (97–98% fructose after hydrolysis). The levan from each medium was thoroughly purified, and several samples of the products from 2 and 10% (w/v) sucrose media were prepared.

Leuconostoc dextranicum when cultured in media A and B containing 10% (w/v) sucrose produced large quantities of dextran (96–98% glucose) while *L. mesenteroides*, being apparently more fastidious in its requirements, produced dextran in medium B only. After ethanolic precipitation the dextran from *L. dextranicum* was insoluble in water, while the product from *L. mesenteroides* gave a colloidal suspension similar to that of levans. However, when the material was worked into a paste and then diluted by adding more water a suspension was obtained which was fairly stable on standing, but precipitation occurred when it was centrifuged at a low speed.

Aggregation of soil particles

The ability of each product to form water-stable soil crumbs was estimated. Results are presented in Figs. 1, 2 and 3. The amounts of the different levans and dextrans applied to soil are expressed as a percentage of the air-dried soil and the plotted values of percentage aggregation are the means of duplicate results, except in the case of levans 107 and F7 where only single estimations were made. For substances giving an average aggregation of 10–80%, S.E. (single determination) = ± 2·46, and S.E. (mean of 2 determinations) = ± 1·74. For aggregations less than 10% or greater than 80%, S.E. falls towards zero.

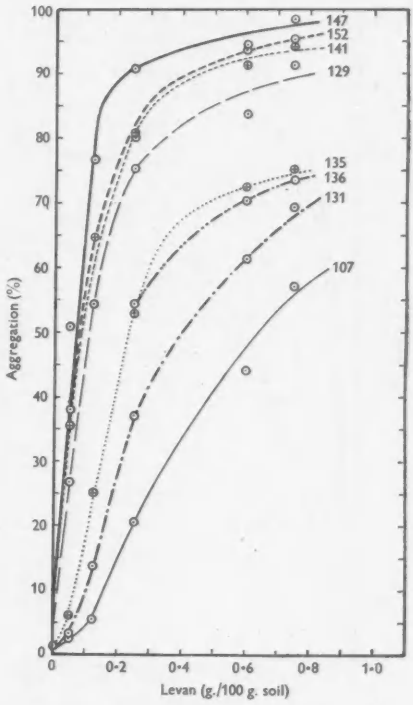

Fig. 1. Effect of increasing quantities of various levans on aggregation of soil particles. (Levans produced by *B. subtilis* when cultured in medium A containing from 2 to 10% sucrose.)

Levan preparation	Culture medium
147, 152, 129	A + 2% (w/v) sucrose
141	A + 4% (w/v) sucrose
136	A + 6% (w/v) sucrose
135	A + 8% (w/v) sucrose
131, 107	A + 10% (w/v) sucrose

Up to 0·25%, the aggregating effect bears an approximately linear relationship to the amount of levan or dextran added. Consequently, 0·25% appears to be the most suitable concentration at which to appraise the aggregating effect of the different products.

All the levans and dextrans had a marked ameliorative effect on the physical condition of soil, but some levan products had a more pronounced effect than others. Apparently the aggregating effect of the levan decreases with increase in sucrose content of the medium. Also it appears that, when two or

more samples of levan are prepared from the same medium but from different lots of culture solution, their aggregating effects are not exactly alike.

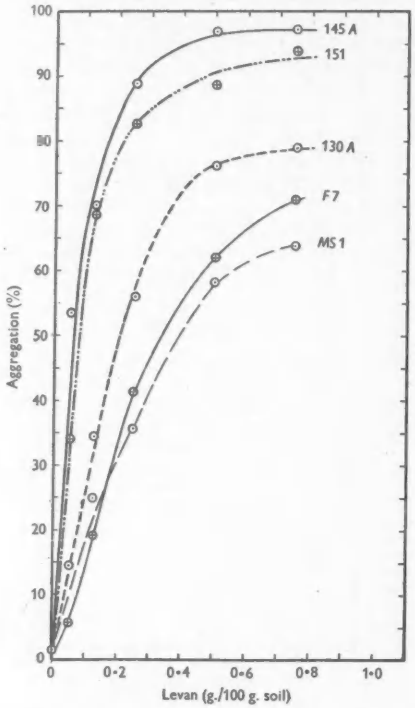

Fig. 2. Effect of increasing quantities of various levans on aggregation of soil particles. (Levans produced by *B. subtilis* when cultured in medium B containing 2 and 10% sucrose.)

Levan preparation	Culture medium
130 A, 145 A	B + 2 % (w/v) sucrose
MS 1, F 7, 151	B + 10 % (w/v) sucrose

Effect of applying large doses of levan to soil. In the case of those levans which possess a relatively low aggregating effect, it was thought that the degree of aggregation might be increased by applying larger quantities of levan to the soil. This was investigated (Table 1) by treating samples of soil with product 131, until the soil contained 2 and 5% levan, and comparing the degree of aggregation produced with that obtained when the soil contained from 0·05 to 0·75% levan. All determinations were done in duplicate and mean results are shown.

Apparently, further application of levan beyond a certain point has very little effect on aggregation of the soil particles, which suggests that soil can only absorb a certain amount of levan, and, having absorbed this amount, the degree of aggregation produced is dependent on the activity of the substance.

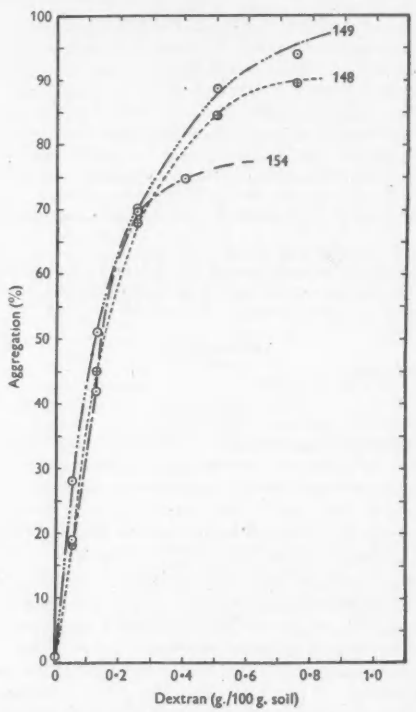

Fig. 3. Effect of increasing quantities of various dextrans on aggregation of soil particles.

Dextran preparation	Synthesizing organism	Culture medium
148	*Leuconostoc dextranicum*	B + 10 % (w/v) sucrose
149	*Leuconostoc dextranicum*	A + 10 % (w/v) sucrose
154	*Leuconostoc mesenteroides*	B + 10 % (w/v) sucrose

Table 1. *Effect of applying levan to soil in large and small amounts*

Levan (no.)	Amount added to soil (%)	Aggregation (%)
131	5·0	76·1
	2·0	65·6
	0·75	69·4
	0·50	61·4
	0·25	37·0
	0·125	13·6
	0·05	3·3
	Control	0·8

Table 2. *Effect of medium and of percentage sucrose in the medium on the intrinsic viscosity and molecular weight of levans produced by* B. subtilis

($K_m = 5 \times 10^{-4}$)

Polysaccharide (no.)	Medium in which polysaccharide was produced	Concentration c (in primary moles)	Flow time (sec.)	Density d	$\frac{\eta_{sp}}{c}$	Mol. wt.
147	A+2% (w/v) sucrose	0·093	286·6	1·007	36·2	26,800
		0·062	158·8	1·005	23·0	
		0·031	97·5	1·003	15·5	
		0·0155	79·6	1·001	13·4	
		0·0093	74·2	1·000	13·4	
			(Water = 66·0)			
152	A+2% (w/v) sucrose	0·093	169·6	1·007	17·0	18,800
		0·062	117·3	1·005	12·7	
		0·031	86·5	1·003	10·13	
		0·0155	75·8	1·001	9·68	
		0·0078	70·9	1·000	9·48	
			(Water = 66·0)			
129	A+2% (w/v) sucrose	0·093	112·4	1·007	14·30	16,000
		0·062	82·4	1·005	11·43	
		0·031	61·6	1·003	9·00	
		0·0155	54·3	1·001	7·79	
		0·0093	51·5	1·000	8·38	
			(Water = 48·5)			
141	A+4% (w/v) sucrose	0·093	131·0	1·007	10·73	14,000
		0·062	99·8	1·005	8·40	
		0·031	80·6	1·003	7·26	
		0·0155	73·4	1·001	7·28	
		0·0093	70·2	1·000	6·90	
			(Water = 66·0)			
136	A+6% (w/v) sucrose	0·093	71·1	1·007	5·12	8,000
		0·062	62·2	1·005	4·70	
		0·031	54·7	1·003	4·23	
		0·0204	52·4	1·002	4·00	
			(Water = 48·5)			
135	A+8% (w/v) sucrose	0·093	67·5	1·007	4·31	7,200
		0·062	60·2	1·005	4·00	
		0·031	53·9	1·003	3·71	
		0·0155	51·3	1·001	3·80	
			(Water = 48·5)			
107	A+10% (w/v) sucrose	0·083	56·4	1·006	4·42	6,400
		0·062	50·1	1·005	3·44	
		0·031	45·6	1·003	3·29	
			(Water = 41·5)			
131	A+10% (w/v) sucrose	0·093	64·6	1·007	3·67	6,200
		0·062	58·6	1·005	3·45	
		0·031	53·0	1·003	3·10	
		0·0155	50·8	1·001	3·10	
			(Water = 48·5)			
145A	B+2% (w/v) sucrose	0·093	134·5	1·007	19·3	18,000
		0·062	90·1	1·005	14·0	
		0·031	64·3	1·003	10·65	
		0·0155	55·1	1·001	8·71	
		0·0093	52·8	1·000	9·46	
			(Water = 48·5)			
130A	B+2% (w/v) sucrose	0·093	101·5	1·007	11·80	15,600
		0·062	79·8	1·005	10·53	
		0·031	61·1	1·003	8·48	
		0·0155	54·2	1·001	7·68	
		0·0093	52·1	1·000	7·96	
			(Water = 48·5)			
MS1	B+10% (w/v) sucrose	0·093	76·8	1·007	6·39	9,400
		0·062	65·6	1·005	5·81	
		0·031	55·9	1·003	5·00	
		0·0155	52·0	1·001	4·77	
			(Water = 48·5)			
F7	B+10% (w/v) sucrose	0·083	65·5	1·006	7·15	9,600
		0·062	55·8	1·005	5·66	
		0·031	47·8	1·003	5·00	
			(Water = 41·5)			
151	B+10% (w/v) sucrose	0·093	121·8	1·007	9·23	12,200
		0·062	97·2	1·005	7·74	
		0·031	78·9	1·003	6·42	
		0·0155	72·2	1·001	6·13	
			(Water = 66·0)			

Table 3. *Intrinsic viscosity and molecular weight of the dextran produced by* Leuconostoc mesenteroides

($K_m = 5 \times 10^{-4}$)

Dextran (no.)	Medium in which dextran was produced	Concentration c (in primary moles)	Flow time (sec.)	Density d	$\frac{\eta_{sp}}{c}$	Mol. wt.
154	B + 10% (w/v) sucrose	0·111	185·6	1·009	16·5	14,000
		0·055	100·7	1·004	9·65	
		0·028	81·1	1·003	8·29	
		0·014	72·5	1·001	7·14	
		0·007	69·2	1·000	7·00	
			(Water = 66·0)			

Viscosity measurements

Since the aggregating effects of levans from different media were not alike, it was thought that the ability to stabilize soil crumbs might be related to some chemical or physical property of the polysaccharides. This idea was supported by the observation that after ethanolic precipitation the products from '2% (w/v) sucrose' media were tough and when stretched showed pronounced recoil, while those from '10% (w/v) sucrose' medium were viscous and capable of being drawn into threads. The chain length of the polymers, which influences their physical properties, was therefore investigated by viscosity measurements.

The intrinsic viscosities of solutions of the various levans and the dextran produced by *L. mesenteroides* were determined, and the values correlated with the corresponding aggregating effects. Whilst the value $\lim_{c \to 0} \left(\frac{\eta_{sp}}{c}\right)$ yielded all the information required, the molecular weights were found from the Staudinger relationship $\frac{\eta_{sp}}{c} = K_m M$, where K_m is a constant estimated to have the value 10^{-3} for cellulose in Schweitzer's reagent ($c = 1{\cdot}4\%$ (w/v) (Staudinger, 1933)). More recently the constant $K_m = 5 \times 10^{-4}$ has been used by Staudinger (1938).

The constants for our levans and dextran were not determined and $K_m = 5 \times 10^{-4}$ was selected. The use of a constant which applies to cellulose in Schweitzer's reagent in calculating the molecular weights of levans and dextrans is admittedly an approximation but gives information on the order of the molecular weights. The results are shown in Tables 2 and 3.

The intrinsic viscosities and hence the molecular weights of the levans decreased with increasing sucrose content of the medium. In the case of '2% sucrose' media the form of nitrogen supplied to the organism did not materially influence the intrinsic viscosities of the products. The intrinsic viscosity of the dextran produced by *L. mesenteroides* is similar to that of some levans.

There appears to be a relationship between the value of $\lim_{c \to 0} \left(\frac{\eta_{sp}}{c}\right)$ and the percentage aggregation. From the values for intrinsic viscosity and aggregating effect of 13 levans and 1 dextran a correlation coefficient was calculated and found to be 0·809. This value indicates a significant correlation (odds 1 in 1000).

A dot diagram, in which the value of $\lim_{c \to 0} \left(\frac{\eta_{sp}}{c}\right)$ is plotted against the degree of aggregation produced when soil contains 0·25% of the various levans and dextrans, is given in Fig. 4. No curve is fitted to the

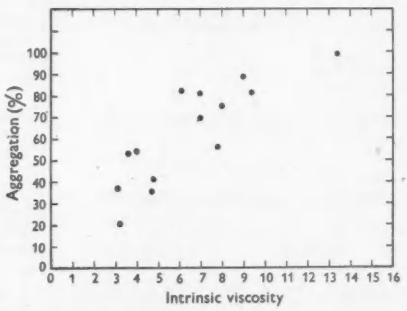

Fig. 4. Relationship between the intrinsic viscosities and the aggregating effects of 13 levans and 1 dextran.

points on the diagram; this cannot be done satisfactorily until the shape of the curve as it approaches zero and 100% aggregation can be forecast for substances of lower and higher intrinsic viscosities than those so far considered.

Nitrogen content of levans and dextrans

Solutions of bacterial polysaccharides are very viscous and separation of the metabolic products from bacterial cells is very difficult. Levans and dextrans as usually prepared contain a small amount of nitrogen (0·2–0·5%) which may be present as an impurity or as a constituent of the polysaccharide

molecule. Stacey (1943) has suggested that dextrans consist of polyglucose chains linked together by units of the synthesizing enzyme which remains as an integral part of the complex mucopolysaccharide. In our earlier investigations we found that the levan obtained when *B. subtilis* was cultured in '10% sucrose' media contained 0·09% N, whereas that from '2% sucrose' media contained 0·2 to 0·3% N, and since the product from the '10% sucrose' media was not as effective an aggregator of soil as the product from '2% sucrose' medium it appeared that the aggregating effect was related to the nitrogen content. However, by improving the technique used for separating the polysaccharides from the bacterial cells, the nitrogen content of the products was lowered appreciably without degrading the products and without lowering the aggregating effects. In Table 4 are shown the nitrogen content, molecular weight and aggregating effect of some of our most purified levans and dextrans.

Table 4. *Nitrogen content, molecular weight and aggregating effect of some levans and dextrans*

Polysaccharide (no.)	Medium in which polysaccharide was formed	Nitrogen (%)	Mol. wt.	Aggregating effect when soil received 0·25% polysaccharide
Levan				
147	A + 2% (w/v) sucrose	0·12	26,800	90·9
152	A + 2% (w/v) sucrose	0·13	18,800	80·1
129	A + 2% (w/v) sucrose	0·05	16,000	75·1
130A	B + 2% (w/v) sucrose	0·18	15,600	56·0
145A	B + 2% (w/v) sucrose	0·09	18,000	88·9
141	A + 4% (w/v) sucrose	0·09	14,000	80·9
136	A + 6% (w/v) sucrose	0·06	8,000	54·1
135	A + 8% (w/v) sucrose	0·03	7,200	52·9
131	A + 10% (w/v) sucrose	0·02	6,200	37·0
107	A + 10% (w/v) sucrose	0·10	6,400	20·5
MS1	B + 10% (w/v) sucrose	0·10	9,400	35·8
F7	B + 10% (w/v) sucrose	0·09	9,600	41·4
151	B + 10% (w/v) sucrose	0·06	12,200	82·7
Dextran				
148	B + 10% (w/v) sucrose	0·22	—	67·9
149	A + 10% (w/v) sucrose	0·00	—	70·1
154	B + 10% (w/v) sucrose	0·11	14,000	69·7

It appears that the aggregating effects and the molecular weights of these products are not related to their nitrogen contents. This is most evident in the case of levans from the same medium.

In the case of levans 141, 136, 135 and 131 there appears to be a decline in molecular weight and aggregating effect as the nitrogen content of the products decreases, but these materials are from media containing different concentrations of sucrose, and it has been shown that the molecular weight and aggregating effect decrease with increasing sucrose concentration of the medium. The decline in nitrogen content, accompanying the decrease in molecular weight and aggregating effect, is possibly due to the fact that the yield of levan/l. of culture solution increases with increase in sucrose content of medium A. This increase in yield of levan is accompanied by a lowering of the nitrogen content of the 'crude levan', which suggests that the ratio of levan to bacterial cells in the culture solution is increased with increase in sucrose content of the medium. Microscopical examination of levan solutions has shown that, even after most careful purification, a small amount of bacterial cells remains. It is reasonable to expect that the amount of bacterial cells that remains is influenced by the ratio of levan to bacterial cells in the crude material.

In another experiment levans were isolated in crude form and samples of the crude material were purified to different degrees. The nitrogen content and molecular weight of each product were determined. Results are recorded in Table 5.

Table 5. *Nitrogen content and molecular weight of 'crude' and 'purified' levans*

Levan (no.)	Nitrogen content (%)	Molecular weight
151 Crude	0·13	12,200
Purified	0·06	12,200
139 Crude	0·43	6,200
Purified	0·11	7,600

Relatively large amounts of nitrogen were removed without degrading the substances, and it appears that there is no correlation between molecular weight and nitrogen content of levans produced by *B. subtilis*.

Table 6 shows the results of an experiment designed to test how the aggregating effect of a levan was affected when its nitrogen content was lowered.

Table 6. *Relation between aggregating effect and nitrogen content of a levan*

Levan (no.)	Amount added to soil (%)	Aggregation (%) Sample 1, N = 0·25%	Sample 2, N = 0·09%
124	0·75	73·8	83·3
	0·50	69·8	78·6
	0·25	48·8	60·8

As the nitrogen content decreased the aggregating effect increased, which shows that purification removed only extraneous matter and did not affect the ability of the substance to form water-stable soil crumbs.

It will be recalled that it has been suggested that polysaccharides owe certain of their immunological properties to the presence in them of a nitrogenous constituent, e.g. Fitzgerald (1933) found from serological studies that the antigenic activity of a polysaccharide produced by *Leuconostoc mesenteroides* disappeared when its nitrogen content was reduced to less than 0·2%. However, Evans, Hawkins & Hibbert (1941) found that anti-*L. mesenteroides* sera (produced in rabbits) gave precipitin reactions with relatively high dilutions of the homologous polysaccharide, dextran, having a maximum nitrogen content of 0·08%. This suggests that the small amount of nitrogen present in the dextran may not be responsible for the immunological reactions of the polysaccharide.

When our levans were treated with hot dilute NaOH, a flocculent precipitate appeared in the solution, and when it was removed a degraded levan was recovered from the solution which had a reduced nitrogen content and a very low aggregating effect (cf. Stacey, 1943). A similar precipitate was formed when solutions of the levan from *B. subtilis* were dialyzed. The precipitate settled to the bottom of the dialysis tube and was easily removed. Microscopic examination showed that it consisted, almost entirely, of bacterial cells. The levan recovered from the dialyzed solution had a much lower nitrogen content and a slightly higher aggregating effect. Apparently, the effect of NaOH on levan solutions is twofold: it facilitates removal of nitrogen by precipitating the bacterial cells and 'cell debris', and it degrades the levan. It is thought that these are separate actions, and it is not necessary to degrade the levan to remove nitrogen.

Since the intrinsic viscosities, and hence the molecular weights of levans and dextrans, were not affected by removal of nitrogen, it is concluded that the nitrogen content of the polysaccharides examined is due to the presence of bacterial cells, as impurities, in the substances.

Stability of soil aggregates made with bacterial levan

It is well known that unstable soil aggregates disintegrate when they are placed in water. However, when aggregates containing 0·5% levan were submerged in water for 20 days it was observed that they were more or less unchanged. They had absorbed a large amount of moisture but showed no sign of crumbling.

The data in Table 7 show that the method of wetting aggregates containing levan does not materially influence their stability.

Table 7. *Effect of maintaining aggregates in a moist condition for long and short periods before wet sieving*

Levan (no.)	Amount added to soil (%)	Aggregation (%) Aggregates absorbing moisture by capillary action for 17 hr. before wet sieving	Aggregates submerged for 30 min. before wet sieving
151	0·5	84·7	88·5
	0·125	54·4	68·8

In another experiment aggregates containing 0·5% levan (product 151) were dropped into boiling water and held at a temperature of 95° for 10 min. with and without stirring. They were then cooled and the degree of aggregation measured by the wet-sieving technique. It is rather remarkable that, without stirring, a large proportion (over 65%) of the aggregates remained stable after heating, and that, with stirring, as much as 35% still remained stable.

Soil aggregates, containing 0·5% levan, were broken down when shaken in water for 1 hr. However, when the fine soil was removed from the shaking bottle, dried to the 'sticky point' and pushed through a 3 mm. sieve, water-stable aggregates were reformed. Approximately 40% of the polysaccharide applied to the soil was present in the water with which the soil was shaken. These results suggest that a certain amount of levan is fixed on soil particles.

Table 8 gives the results of an experiment designed to discover if levan, present on soil particles, was resistant to microbial decomposition. Aggregates containing 0·5 and 0·125% levan were moistened with a soil suspension and incubated at 27°. At intervals samples were removed and the percentage of water-stable aggregates estimated.

It is concluded that levan present on soil particles is slowly decomposed by micro-organisms.

Table 8. *Effect of microbial activity on stability of 'levan-soil aggregates'*

Levan (no.)	Amount added to soil (%)	Aggregation (%) Before incubation	After 5 days' incubation	After 11 days' incubation	After 31 days' incubation
151	0·5	88·5	82·8	61·0	44·1
	0·125	68·8	23·7	8·2	7·2

DISCUSSION

Polysaccharides are normal metabolic products of micro-organisms. Numerous species of bacteria, fungi and actinomyces are capable of producing a wide variety of complex polysaccharides as cell constituents or as extracellular products. Hence it is reasonable to expect that, under natural conditions, the diverse flora of the soil may synthesize many polysaccharides from the constituents of vegetable material, and that these substances may be of considerable importance in the formation of crumb structure in soil.

Polysaccharides are probably only one of the groups of microbial metabolic products having an ameliorative effect on soil structure, and only when further knowledge is available will it be possible to estimate the full extent to which biological activity influences the physical condition of soil.

SUMMARY

1. It has been shown that bacterial polysaccharides of the levan and dextran types exert a pronounced binding effect on soil particles.

2. *Bacillus subtilis* was cultured in a number of different media, in which the sucrose concentration and source of nitrogen were varied, and it was observed that the yield of levan increased with increase in sucrose content of the medium from 2 to 10% (w/v). The aggregating effect of the levan decreased, however, with increasing sucrose content of the medium, and was paralleled by a decrease in intrinsic viscosity and molecular weight of the levans.

3. It appears that the nitrogen content of levans and dextrans is due to the presence of bacterial cells, as impurities, in the substances. There was no correlation between aggregating effect and nitrogen content, or between molecular weight and nitrogen content of either levans or dextrans.

4. Soil crumbs, containing small amounts of levan, are extraordinarily stable to the dispersing actions of water, heat and shaking, but are eventually broken down by the activities of micro-organisms.

5. Preliminary investigations indicate that levans are fixed on soil particles.

6. It is not suggested that polysaccharides are the sole agents concerned in aggregating soil, but it does appear that they may be of considerable importance.

Grateful acknowledgements are due to Mr P. A. Collier for statistical analysis of the results and to Dr A. H. Lewis for helpful advice.

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Aggregate Formation in Soil

2. INFLUENCE OF VARIOUS CARBOHYDRATES AND PROTEINS ON AGGREGATION OF SOIL PARTICLES

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Geoghegan & Brian (1948) reported that bacterial polysaccharides of the dextran and levan types had a marked aggregating effect on soil particles. Further, it was observed that aggregating effect was related to the intrinsic viscosity and molecular weight of the substances. These results suggest that other viscous substances of high molecular weight may have an ameliorative effect on soil structure.

EXPERIMENTAL

The aggregating effect of various carbohydrates and proteins was determined by the method of Geoghegan & Brian (1948).

RESULTS

Results are presented in Table 1. The amounts of the different substances applied to soil are expressed as percentages of the air-dried soil and the figures quoted for aggregation are the means of duplicate results.

Table 1. *Influence of various carbohydrates and proteins on aggregation of soil particles*

Substance	Percentage aggregation* when 0·25% of the substance was applied to soil
Crude polysaccharide from a strain of *Penicillium luteum*	50·1
'Cellofas W.L.D.'	45·5
'Cellofas W.F.Z.'	25·6
Quince gum	25·5
Pectin	3·4
Inulin	0·8
Egg albumin	32·9
Trypsin	4·5
Pepsin	1·2
Pancreatin	1·1
Control	1·2

* For substances giving an aggregation greater than 10%, S.E. for the mean of two determinations = ±1·09.

DISCUSSION

The crude polysaccharide from *Penicillium luteum* had a definite aggregating effect, although less than that of levans and dextrans (cf. Geoghegan & Brian, 1948). This indicates that under natural conditions fungal polysaccharides, like bacterial polysaccharides, may play an important role in soil structure formation.

The results obtained with egg albumin, trypsin, pepsin and pancreatin suggest that some proteins are capable of aggregating soil particles. Proteins from plants and micro-organisms are present in soil organic matter, and it is possible that some of these substances may help in the formation of water-stable soil crumbs.

It is interesting to note that 'Cellofas W.F.Z.', although more viscous than 'Cellofas W.L.D.', is less effective as an aggregator; the former is the sodium salt of carboxymethyl cellulose and the latter an ether of cellulose.

Inulin, which is composed of anhydrofructofuranose units, the linkages being at positions 1 and 2 of the fructose chain, is unable to aggregate soil. This is not surprising, because solutions of this substance have low viscosities. Haworth (1936) claims that the inulin molecule consists of a laminated series of fructofuranose rings which are not joined end to end as in the case of cellulose or starch, and that this spatial arrangement is responsible for the low viscosity of solutions of the substance.

Pectin, the specific viscosity of which is greater than that of levans from *Bacillus subtilis*, failed to aggregate soil, and quince gum which is still more viscous only caused 25% aggregation. This is important as it shows that, while substances capable of aggregating soil have high viscosities, there is no reason to expect that all viscous substances will improve soil structure.

Work along these lines will provide information on the relative powers of different substances to aggregate soil particles, and will help to elucidate the relationship between chemical structure and the ability of a substance to form water-stable soil crumbs.

SUMMARY

The aggregation of soil particles by viscous substances other than levans and dextrans has been assessed. Results show that while some biological products have a moderate aggregating effect it does not follow that all viscous substances will improve soil structure.

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Antipyretic Action and Catalase Activity

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Antipyretics are well known as stabilizers of commercial hydrogen peroxide solutions (Kausch, 1938; Slater, 1945), and it has been suggested by Bailey (1937) that 'antipyretic substances are in general aromatic, many of them phenolic, and quite possibly lower the temperature of the body by reducing the rate of oxidation', conceivably by interfering with H_2O_2 metabolism. In tissue metabolism, H_2O_2 may be produced by reactions involving, for example, D-amino-acid oxidase, xanthine oxidase, uricase (Sumner & Somers, 1943), and the chemical identification of H_2O_2 as a metabolic product has recently been demonstrated by Tosic & Walton (1946). The H_2O_2 produced is decomposed by the widely distributed cellular enzyme catalase, but simple protective decomposition of H_2O_2 into O_2 and water is now considered by Keilin & Hartree (1945) to be less likely than catalysis of coupled oxidation of alcohols, and possibly of other biologically important compounds.

The possibility that antipyretic action may not be wholly central in origin is suggested by the hyperthermic action of dinitro-α-naphthol in a dog deprived of its central thermoregulatory mechanism (Heymans, 1928), and by the antipyretic action of acetanilide, quinine and pyramidone in decerebrate rabbits exhibiting pyrexia induced by β-tetrahydronaphthylamine (Skowronski, 1929). The antipyretic substances, sodium salicylate, quinine and pyramidone, have a depressant action on isolated-tissue respiration (Nitzescu & Cosima, 1923), and the reduction of methylene blue by liver tissue is inhibited by antipyretics such as aspirin, antipyrine, phenacetin, quinine and sodium salicylate, as well as by a number of typical antioxidants (Boutaric & Jacquinot, 1931).

Antipyretic activity is broadly associated with particular chemical structures, and several representative antipyretic substances were tested for inhibitory activity in the isolated catalase-H_2O_2 system, in an attempt to provide evidence for the hypothetical mechanism suggested above for possible peripheral action of antipyretics. Of the four main types of antipyretic substances, acetanilide and phenacetin represent typical aniline derivatives, salicyclic acid and acetylsalicylic acid represent typical phenol derivatives, antipyrine is a representative pyrazolone derivative, and quinine represents a number of quinoline derivatives. *p*-Hydroxybenzoic acid is a phenol derivative not ordinarily used as an antipyretic.

EXPERIMENTAL

A horse liver catalase extract, prepared by the method of Sumner, Dounce & Frampton (1941), had a 'Katalase *f*' value (determined by the method of von Euler & Josephson, 1927) of 2560. The catalase content was calculated by colorimetric estimation of the Fe content, by the method of Delory (1943), assuming 0·062% Fe in the dry enzyme (von Euler, Zeile & Hellström, 1930).

The test substances used were A.R. grade, further purified as follows: acetanilide, phenacetin, aspirin, salicylic acid and *p*-hydroxybenzoic acid (recrystallized from conductivity water), quinine (recrystallized from pure benzene), antipyrine (recrystallized from pure toluene). The pH of the Sorensen buffer was checked electrometrically. All solutions were prepared in glass-distilled water, and dust was excluded as far as possible.

Reaction rates were determined at 37° using the Barcroft manometer. The right-hand manometer flask contained 2 ml. of 0·32 M-H_2O_2 (M.A.R.), and 2 ml. of 0·0067 M-phosphate buffer, pH 6·8; the left-hand flask, 2 ml. of 0·32 M-H_2O_2, 1 ml. of 0·0067 M-buffer, 1 ml. of a solution of the test substance, and a Keilin pipette holding 0·16 ml. of undiluted catalase extract. For the calculation of percentage inhibitions, reaction rates were compared with that of the control, in which the amount of O_2 evolved over a period of 5 min. was taken as a measure of initial reaction velocity.

RESULTS

The percentage inhibitions obtained with the substances tested are given in Table 1. The observed inhibitions with the hydroxybenzoic acid derivatives can be due only in negligible degree to the slight lowering of pH, and probably result from the presence of phenolic OH groupings. The latter may interrupt the chain reaction, postulated by Sumner (1941), which involves the formation of the free radicals OOH and OH. A number of workers (e.g. Richter, 1934; Alyea & Pace, 1933; Yakushiyi, 1937; Collier, 1940) have shown, in fact, that catalase is inhibited by phenolic compounds. Collier has shown that, in general, catalase is inhibited by phenols, oximes and amino derivatives which may give rise to HO.NH groupings on oxidation, and that these compounds produce methaemoglobin from haemoglobin, reduce Folin's phenol reagent,

Table 1. *Inhibition by test substances of the decomposition of* 0·16M-H_2O_2 *by catalase*

Conc. of antipyretic (M × 10^{-3})	Percentage inhibitions						
	Acetanilide	Phenacetin	Salicylic acid	Acetyl-salicylic acid	*p*-Hydroxy-benzoic acid	Antipyrine	Quinine
0·4	—	—	—	—	—	—	0
1·1	—	0	44	17	—	0	—
1·8	0	—	—	—	47	—	—
2·3	—	—	76	42	—	—	—
2·6	—	—	—	—	—	0	—
3·5	0	—	—	—	68	—	—
4·5	—	—	89	70	—	—	—
5·3	—	—	—	—	—	0	—
7·0	0	—	—	—	—	—	—
10·5	—	—	—	—	—	0	—
14·0	28	—	—	—	—	—	—

give colour reactions with $FeCl_3$ and resemble antioxidants.

Experiments with concentrations higher than those recorded for phenacetin and quinine were excluded by the low solubility of these compounds. With antipyrine there is obviously no correlation with antipyretic activity, and inhibition with acetanilide was only slight at relatively high concentrations. It is thus not possible to associate antipyretic activity in every case with the *in vitro* decomposition of H_2O_2 by catalase.

SUMMARY

1. Since many antipyretics can be used to stabilize H_2O_2 solutions, it has been suggested that they may also conceivably interfere with H_2O_2 metabolism. To test this theory, a number of representative antipyretics have been tested for inhibitory activity in the isolated enzyme system, catalase-H_2O_2.

2. Inhibition was observed with some, but not all, of the substances tested, i.e. there was no parallelism with antipyretic activity.

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Note on the Manometric Determination of Succinic and α-Ketoglutaric Acids

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Small quantities of succinic and α-ketoglutaric acids, from 0·2 mg. upwards, are now commonly determined manometrically with the help of succinic dehydrogenase (Szent-Györgyi & Gözsy, 1935; Krebs, 1937, 1938). The object of this paper is to draw attention to two sources of errors which we have recently encountered.

Inhibition of succinic dehydrogenase by phosphotungstates

In some determinations of succinate it was noted that the ethereal extract contained a powerful inhibitor of succinic dehydrogenase, resulting in delayed or incomplete oxidation of the succinate. This was found to be phosphotungstate which arises from the tungstate added as a deproteinizing agent and from phosphate present in the tissue preparations. Phosphotungstic acids are known to be readily extractable with ether.

Whilst Na tungstate (2×10^{-2}M) has no effect on succinic dehydrogenase, Na phospho-18-tungstate ('*A*-acid' of Wu, 1920) is a strong inhibitor in relatively low concentrations. For example $0{\cdot}5 \times 10^{-4}$M caused an inhibition of 64% (pH 7·4; succinate concentration 0·01M; 40°). Na phospho-24-tungstate, also prepared according to Wu (1920), is likewise an inhibitor, but higher concentrations, about five times that of the 18-acid, are required to produce the same effect.

These inhibitions might be expected as it is known that succinic dehydrogenase possesses SH groups (Hopkins & Morgan, 1938), and that phosphotungstates readily oxidize SH compounds to the disulphides (Folin & Marenzi, 1929; Schöberl & Ludwig, 1937; Schöberl & Rambacher, 1938). The inhibition of succinic dehydrogenase depends on the concentration of succinate. This again is not unexpected since Hopkins, Morgan & Lutwak-Mann (1938) have shown that succinate protects the SH groups of the enzyme against other reagents.

When solutions of tungstate which do not contain phosphate are acidified, practically all the tungstic acid is precipitated, but in the presence of phosphate, tungstic acid in excess of that reacting with protein and similar substances remains in the solution as complex phosphate. The formation of inhibitory quantities of phosphotungstate can easily be prevented by avoiding an excess of tungstate. A minimum amount, ascertained on a sample of the material, is recommended. We find that 4 ml. of a neutralized tissue suspension containing 0·4 g. of fresh tissue and diluted to 15 ml. with water or washings is satisfactorily deproteinized by the addition of 0·25 ml. of 10% (w/v) Na tungstate and subsequent acidification with H_2SO_4 (blue to congo red). We retain tungstate as a deproteinizing agent because, unlike other deproteinizing agents, it yields filtrates which do not froth when ether is bubbled through the solution at a rapid rate.

Interference of glutamic acid in the determination of α-ketoglutaric acid

$KMnO_4$ in acid solution is used for the oxidation of α-ketoglutaric to succinic acid, and it has been pointed out previously (Krebs, 1938) that several substances which may occur in biological material, such as α-hydroxyglutarate and arginine, also yield succinate when treated with the reagent. When these substances are present α-ketoglutarate must be separated prior to the oxidations. This can be effected by the conversion into the 2:4-dinitrophenylhydrazone and ethereal extraction of the latter. In the absence of interfering material a simplified procedure may be adopted, i.e. direct oxidation with $KMnO_4$ of the deproteinized solution (Krebs, 1938; Krebs & Eggleston, 1940).

In recent experiments we found that under some conditions glutamic acid, which is frequently present in biological material, yields succinate on treatment with $KMnO_4$. It is noteworthy that in pure solution glutamic acid does not react significantly. Previous findings on this point (Krebs, 1938) were confirmed by recent tests; it was further found that no oxidation of glutamic acid occurred in mixtures of glutamic acid with α-ketoglutaric acid, or glucose, or pyruvate, or lactate and several amino-acids. But in deproteinized homogenates of liver and other tissues added glutamic acid was found to yield varying amounts of succinate, the maximum being 70% of the theoretically possible value. Deproteinized suspensions of *minced* liver had little or no effect on the oxidation of glutamic acid.

We have not been successful in identifying the substance in liver and other tissue homogenates responsible for the oxidizability of glutamic acid by acid $KMnO_4$. The following substances added to a pure solution of glutamic acid had no effect on the oxidizability: $CuSO_4$, $FeSO_4$, $MnSO_4$, haemin, the substances of rabbit bile, the amino-acids contained in casein hydrolysate.

In view of the widespread occurrence of glutamic acid in biological material the direct treatment of deproteinized solutions with permanganate must be deprecated. We separate α-ketoglutaric from glutamic acid by extraction of the strongly acidified solution with ethyl ether, using a continuous extractor of the Kutscher-Steudel type with the dimensions described by Krebs, Smyth & Evans (1940). The deproteinized filtrate is acidified with 0·1 vol. of 50% (v/v) H_2SO_4. The rate of extraction is illustrated by the following data. When 5·84 mg. of α-ketoglutaric acid, dissolved in 15 ml. of 2N-H_2SO_4 were extracted, 71% appeared in the extract within 15 min., 90% within 30 min., 95% within 60 min. These figures depend of course on many factors, especially the design of the apparatus and the rate at which ether passes through the solution; it is therefore necessary to check the rate for each set up. In order to have a safety margin we extract for 2 hr. The ether is then evaporated and the residue is dissolved in a few ml. of water. It is usually divided into 2 parts; in one succinate is determined directly, whilst the other is acidified and treated with an excess of $KMnO_4$ as described by Krebs & Eggleston (1945).

This procedure eliminates interference by glutamic acid, glutamine and arginine, but if α-hydroxyglutarate is present the dinitrophenylhydrazone method (Krebs, 1938) remains the method of choice.

SUMMARY

1. Succinic dehydrogenase is inhibited by phospho-18-tungstate and phospho-24-tungstate, presumably because these reagents oxidize the SH groups of the enzyme.

2. Glutamic acid, whilst stable in the presence of potassium permanganate in pure solution, is oxidized by this reagent to succinic acid in the presence of certain deproteinized tissue extracts.

3. The above reactions may cause errors in the manometric determination of succinate and α-ketoglutarate. Procedures are described which avoid such errors.

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New Zealand Fish Oils

5. COMPOSITION OF THE FATS OF THE SCHOOL SHARK (*GALEORHINUS AUSTRALIS*, MACLEAY)

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The school or snapper shark (*Galeorhinus australis*) occurs in both New Zealand and Australian waters and, in common with related species found elsewhere, such as the North West Pacific soupfin shark (*G. zyopterus*) (Byers, 1940), the South American tollo (*G. mento*) (Pfister, 1936*a*, *b*) and the South African soupfin shark (*G. canis* Rond) (Molteno, Rapson, Roux, Schwartz & van Rensburg, 1945) is an important source of vitamin A-rich liver oil (cf. Davies & Field, 1937; Jowett & Davies, 1938; Cunningham & Slater, 1939). Little, however, is known concerning the composition of oils from these species.

In most work on the fatty acid composition of fish oils from a given species, one sample only has been used so that there is no information as to the extent of the variation between individuals. This may be

large, as in New Zealand groper (*Polyprion oxygeneios*) liver oil (Shorland & Hilditch, 1938), or small as in New Zealand ling (*Genypterus blacodes*) liver oil (Shorland, 1939). In this work, therefore, the fats from eight specimens of school shark selected at random from one day's catch in Cook Strait on 16 February 1944 have been studied separately.

EXPERIMENTAL AND RESULTS

Information about the specimens used in this study is given in Table 1.

The sharks were divided by a transverse cut at the posterior gill slit into head, body, liver and rest of viscera. Each of the tissues was treated with twice its weight of 95% ethanol, held at the boiling point for 15 min. and

Table 1. *Details of school sharks used in this investigation*

Sample (no.)	Locality	Length (cm.)	Weight (kg.)	Weight of liver (g.)	Sexual condition
1	Karori Rock (10 fathoms)	164	15·43	568	♀, commencing development
2	Panario patch (60–80 fathoms)	149	13·63	822	♂, undeveloped
3	" "	123	6·80	367	♀, undeveloped
4	" "	136	10·90	908	♀, undeveloped
5	" "	132	9·99	740	♀, undeveloped
6	Island Bay bank (60–80 fathoms)	151	24·95	1250	♂, commencing development
7	" "	144	12·27	568	♀, commencing development
8	" "	138	11·82	1080	♀, commencing development

Table 2. *Proportions of tissues and distribution of fats in New Zealand school shark*

	Head			Body			Viscera other than liver			Liver			
		Oil			Oil			Oil			Oil		
Sample (no.)	% total weight	(%)	% total in fish	% total weight	(%)	% total in fish	% total weight	(%)	% total in fish	% total weight	(%)	% total in fish	% oil in shark
1	21·3	0·2	3·3	67·7	0·5	26·5	7·3	0·6	3·4	3·7	23·1	66·8	1·3
2	21·6	0·2	1·1	66·1	0·5	8·7	6·3	1·0	1·6	6·0	55·9	88·6	3·8
3	21·6	0·3	3·2	66·3	0·5	16·3	6·7	0·6	2·0	5·4	29·6	78·5	2·0
4	17·7	0·1	0·4	69·0	0·5	7·2	5·0	0·7	0·7	8·3	52·7	91·7	4·8
5	18·2	0·4	1·5	69·3	0·7	9·9	5·1	1·2	1·3	7·4	57·6	87·3	4·9
6	10·7	0·1	0·3	78·4	0·4	10·6	5·9	0·8	1·6	5·0	51·8	87·5	3·0
7	23·2	0·2	2·3	66·2	0·3	9·8	6·0	0·9	2·6	4·6	37·6	85·3	2·0
8	19·7	0·2	0·7	65·7	0·5	5·5	5·5	0·9	0·8	9·1	60·7	93·0	5·8
South African soupfin shark	15·3*	0·48	3·5	78·1	0·71	26·8	1·7†	0·7	0·6	4·9	29·2	69·1	2·1

* Excluding foetuses. Recalculated from data given by Molteno *et al.* (1945).
† Intestine only.

Table 3. *Characteristics of New Zealand school shark fats*

	Liver				Body		Head
Sample (no.)	Vitamin A* $\left(\frac{(E^{1\%}_{1\,cm.}\ 328\ m\mu.)}{16}\right)$ (%)	Unsaponifiable matter (%)	Saponification equivalent	Iodine value (Wijs 1 hr.)	Saponification equivalent	Iodine value (Wijs 1 hr.)	Iodine value (Wijs 1 hr.)
1	14·6	31·4	410·0	185·0	366·5	125	112
2	3·6	8·1	317·1	178·0	346·5	146	140
3	0·6	4·5	309·5	183·9	340·5	154	118
4	0·1	2·5	306·5	185·9	328·0	136	103
5	0·4	4·0	307·7	176·8	326·5	136	109
6	2·2	6·4	319·6	170·7	354·0	153	116
7	1·9	8·9	321·0	149·0	301·5	119	137
8	0·6	3·3	308·0	171·7	327·0	127	129

* Spot tests with the Carr-Price reagent showed that some of the body and visceral oils were somewhat richer in vitamin A than cod-liver oil but no positive tests were given by the head oils.

then dried *in vacuo* at a temperature not exceeding 50°. The dried tissues were minced and extracted continuously with light petroleum, b.p. 50–70°, until on replacement with fresh solvent no further lipid was extracted. The final stages of extraction were facilitated by the addition of 5% absolute ethanol to the light petroleum, the ethanolic layer being subsequently withdrawn, evaporated *in vacuo*, re-extracted with light petroleum, and added to the main extract from which the petroleum was distilled, the last traces being removed *in vacuo* on the steam bath.

The proportions of tissues and distribution of fat in the fish are given in Table 2 and the characteristics of liver, body and head fat in Table 3.

As it was not feasible to complete the ester-fractionation analyses of all the samples, a selection was made of the liver oils, while the head and body oils were combined to provide sufficient material for analysis. The head and body fats were further separated into phosphatide and glyceride fractions (cf. Shorland & Hilditch, 1938) yielding 63·6% phosphatide (P = 3·7%) and 36·4% glyceride (P = 0·7%). Qualitative tests showed that the liver oils contained only minor proportions of phosphatide; no separation was therefore made.

The fatty acid composition was determined by the method described by Hilditch (1941) and modified by Shorland & de la Mare (1944). For the purpose of indicating the course of the distillation of the methyl esters the results for school shark liver oil (no. 7) are set out in Table 4 as typical of the general results obtained. The component fatty acids of the fat are listed in Table 5.

DISCUSSION

Rapson, Schwartz & van Rensburg (1945) consider that the composition of the liver fats of marine teleostean fish is largely determined by the extent to which the liver is used as a site for fat storage. They found, in general, that the liver fats of fishes with diffuse oil systems had lower iodine values than the corresponding head, body or intestinal oils, while in the case of fishes with the fat storage localized in the liver the fat from this organ had an iodine value approximating to that of the corresponding body, head and intestinal oil. In the present work it is shown that in common with that of other elasmobranch fish the liver of the school shark is the main site of fat storage (cf. Table 2). Consideration of the iodine values of the liver fats (Table 3) shows that they exceed those of the head and body. Such comparisons of iodine values are complicated by the presence of unsaponifiable matter and by the fact that the head and body lipids are present to a considerable extent as phosphatides. With the methyl esters of the fatty acids, excluding unsaponifiable matter, as basis for comparison, liver oil samples nos. 1, 2, 6 and 7 showed iodine values of 137·3, 152·8, 152·4 and 134·7, respectively, as compared with values of 174·5 and 162·8 for the respective phosphatide and glyceride fractions of the combined head and body oils. The higher iodine value of the head and body oil fatty acids, as

Table 4. *Fractionation of methyl esters of acids from liver fat of school shark no. 7*

	Methyl esters of 'liquid' acids (weight fractionated 39·5 g.)				Methyl esters of 'solid' acids (weight fractionated 11·7 g.)		
No.	(g.)	Saponification equivalent	Iodine value	No.	(g.)	Saponification equivalent	Iodine value
L_1	1·305	246·8	40·1	S_1	0·940	262·9	4·1
L_2	2·929	270·8	78·9	S_2	1·528	255·6	2·1
L_3	3·894	291·8	92·8	S_3	2·647	269·0	2·9
L_4	2·496	295·4	95·7	S_4	2·605	272·0	3·7
L_5	6·549	296·6	99·1	S_5	2·748	297·6	25·4
L_6	4·087	315·0	168·8	S_6	1·088	366·6	61·7
L_7	2·970	322·9	169·6				
L_8	2·953	334·4	257·2				
L_9	2·502	341·6	299·4				
L_{10}	3·241	344·0	308·8				
L_{11}	1·607	347·2	273·7				
L_{12}	1·653	359·3	212·6				
L_{13}	2·971	365·9	183·4				
Total weight of fractions	39·157			Total weight of fractions	11·556		

'Liquid' esters excluding unsaponifiable matter:
L_{12} saponification equivalent 348·2; iodine value 204·0
L_{13} saponification equivalent 359·1; iodine value 177·0

'Solid' esters excluding unsaponifiable matter:
S_6 saponification equivalent 351·6; iodine value 79·2*.

* The relatively high iodine value after resaponification is attributed to the removal of traces of residual phosphatides (cf. Hilditch & Shorland, 1937) which would tend to lower the iodine value in the original fraction.

Table 5. *Component fatty acids of the fats of the school shark*

(*a*) Weight percentages

Liver oil sample	Saturated acids					Unsaturated acids					
	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}
1	1·3	17·1	6·5	1·5	—	0·6 (−2·0 H)	5·4 (−2·0 H)	25·4 (−2·6 H)	20·7 (−4·5 H)	21·5 (−7·6 H)	—
2	1·7	15·3	3·4	1·1	—	1·2 (−2·0 H)	5·9 (−2·0 H)	26·6 (−2·4 H)	19·5 (−5·8 H)	25·3 (−9·6 H)	—
6	2·4	15·2	3·6	—	—	1·1 (−2·0 H)	6·2 (−2·0 H)	31·7 (−2·4 H)	20·2 (−5·4 H)	19·6 (−8·8 H)	—
7	3·9	16·7	5·3	0·1	1·0	0·7 (−2·0 H)	5·3 (−2·0 H)	26·5 (−2·3 H)	15·5 (−4·5 H)	22·8 (−6·5 H)	2·2 (−4·0 H)
Head and body glycerides	0·2	7·8	19·0	5·2	3·4	0·2 (−2·0 H)	5·2 (−2·0 H)	13·5 (−2·2 H)	22·8 (−6·1 H)	22·7 (−12·0 H)	—
Head and body phosphatides	0·3	9·9	13·3	0·3	1·1	1·1 (−2·0 H)	4·3 (−2·0 H)	22·8 (−2·3 H)	16·1 (−6·5 H)	30·8 (−10·0 H)	—

(*b*) Molecular percentages

1	1·7	19·4	6·6	1·4	—	0·7 (−2·0 H)	6·2 (−2·0 H)	26·3 (−2·6 H)	19·6 (−4·5 H)	18·1 (−7·6 H)	—
2	2·2	17·3	3·5	1·0	—	1·5 (−2·0 H)	6·7 (−2·0 H)	27·3 (−2·4 H)	18·4 (−5·8 H)	22·1 (−9·6 H)	—
6	3·1	17·0	3·7	—	—	1·4 (−2·0 H)	6·9 (−2·0 H)	32·2 (−2·4 H)	18·8 (−5·4 H)	16·9 (−8·8 H)	—
7	4·9	18·8	5·4	0·1	0·8	0·9 (−2·0 H)	6·0 (−2·0 H)	27·1 (−2·3 H)	14·5 (−4·5 H)	19·7 (−6·5 H)	1·8 (−4·0 H)
Head and body glycerides	0·3	9·0	19·8	4·9	3·0	0·2 (−2·0 H)	6·0 (−2·0 H)	14·2 (−2·2 H)	22·1 (−6·1 H)	20·5 (−12·0 H)	—
Head and body phosphatides	0·5	11·4	13·7	0·3	1·0	1·4 (−2·0 H)	5·0 (−2·0 H)	23·8 (−2·3 H)	15·5 (−6·5 H)	27·4 (−10·0 H)	—

The quantities in brackets are the values of the mean unsaturation, thus (−4·0 H) indicates an average unsaturation corresponding with two double bonds, but does not necessarily imply the presence only of a diethylenic acid.

Table 6. *Fatty acid composition of school shark liver oil no. 2 before and after hydrogenation*

	Total groups of acids (mol. %)					
	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}
Before hydrogenation	3·7	24·0	30·8	19·4	22·1	—
After hydrogenation	2·8	24·3	33·4	16·7	22·5	0·3
Difference	+0·9	−0·3	−2·6	+2·7	−0·4	−0·3

compared with those of the liver, is in agreement with the views put forward by Rapson *et al.* (1945).

The variation in fatty acid composition between the liver oils (cf. Table 5) reaches a maximum difference in the C_{18} unsaturated acids, where the difference between samples 6 and 1 amounts to 6·3 units weight %. Other similar differences are shown in the C_{20} unsaturated acids, sample 1 containing 5·2 units % more than sample 7, while in the case of the C_{22} unsaturated acids sample 2 is shown to contain 5·7 units % more than sample 6. In assessing differences of this order it is relevant to consider the accuracy of the ester-fractionation method.

Comparison of the original oil with the fully hydrogenated sample (Table 6), shows that by this method in two consecutive analyses differences of up to 2·7 units % may be expected. Using similar comparisons, Hilditch & Houlbrooke (1928), Shorland (1939) and van Rensburg, Rapson & Schwartz (1945) found respectively, differences of up to 1·7, 1·7 and 2·5 units %. Similarly Lovern (1942) found, in duplicate analyses of cod-liver oil, differences between the same fatty acid groups of up to 1·8 units %. By far the most exhaustive and comprehensive analyses carried out on oils, hydrogenated to different iodine values and prepared from the same sample, are those by Hilditch & Terleski (1937) on whale oil and by Harper & Hilditch (1937) on cod-liver oil.

As claimed by these workers (Harper, Hilditch & Terleski, 1937) the final results for the component fatty acids are not necessarily accurate to more than 2 or 3 units % in the case of the higher complex

unsaturated acids. Our interpretation of their claim is that this degree of accuracy has reference to the extent of the deviation from the mean value, so that, when considered from the point of view of the differences likely to be encountered between similar homologous groups of acids in two consecutive analyses, the expected differences are of the order of up to 6 units %. Using the results of the above investigators, the standard deviations of each homologous group of acids have been calculated (Table 7).

Table 7. *Standard deviation of ester fractionation analyses calculated from the data of Hilditch & Terleski* (1937) *and of Harper & Hilditch* (1937)

	Standard deviation from mean of homologous groups of acids (mol. %)					
	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}
Whale oil (6 analyses)	±0·83	±1·54	±1·65	±1·14	±0·75	—
Cod-liver oil (8 analyses)	±0·62	±1·07	±1·04	±1·81	±1·19	±0·39

It is generally considered that, for the results of a single analysis on two different oils to be significant, their difference, for a first approximation, should differ by more than three times the standard deviation of a single observation. In the present series of analyses, because of the relatively small weight of sample used (*c.* 50 g.), it was not expected that the accuracy would be greater than that obtained in the case of the cod-liver oil and whale oil analyses, where larger weights were taken and the fractionation more detailed. However, because of the use in the present work of a more efficient fractionating column as described by Longenecker (1937), the relatively small weight of oil taken for analysis is compensated to some extent by the relatively greater degree of separation of the esters, as compared with that obtained with the Willstätter bulb used in the cod-liver oil and whale oil analyses. It thus seems that the larger differences in the component fatty acids of the school shark liver oils referred to above are possibly significant, but no conclusions regarding the smaller differences appear to be justified.

Comparing the fatty acid composition of the head and body lipids with that of the liver oil (Table 5), it will be seen that the former have appreciably less C_{18} unsaturated acids and palmitic acid but more stearic acid. Attention should also be drawn to the high mean unsaturation of the C_{20} and C_{22} unsaturated acids of the head and body lipids as compared with those of the liver. The differences normally observed between the fatty acid composition of corresponding phosphatides and glycerides (cf. Hilditch & Shorland, 1937), including increased proportions of stearic acid, and C_{20} and C_{22} unsaturated acids in the former as compared with the latter, are not shown in this work.

van Rensburg *et al.* (1945) extended their earlier view that in fishes with diffuse systems of fat storage there is a direct relationship between the iodine values of the head and body oils and the oil contents of these organs, and put forward the hypothesis that these variations correspond to preferential deposition and utilization of C_{20} and C_{22} unsaturated acids, as well as changes in the degree of unsaturation of the acids when the fish are in fat and thin condition respectively. They suggested further that the same may be true of the liver oils of fish where, as in the present case, the liver is the main site of fat storage. In this connexion they showed that, by comparing the fatty acid composition of the liver oils of *Merluccius merluccius* (50% oil in liver) with that of *M. capensis* (28% oil in liver) and *M. gayi* (23% oil in liver), there was a decrease in the amount of C_{20} and C_{22} unsaturated acids from 45·0 to 31·7% with decreasing oil content.

In the present work the oil content of the liver of the school shark has been shown to vary (Table 2) from 23·1% (sample 1) to 55·9% (sample 2), without any marked effect on the fatty acid composition, suggesting that in the school shark it is not a major factor in determining fatty acid composition. It may also be shown (Table 8) that, in other fish where the liver is the main fat depot and the distribution and amount of oil are similar to that of the school shark, the fatty acid composition differs considerably from that of the latter.

The New Zealand ling and school shark referred to in Table 8 have been taken from similar localities in Cook Strait, but the former is distinguished from the latter by containing highly significantly more C_{18} and C_{20} unsaturated acids and highly significantly less C_{22} unsaturated acids in the liver oil. Thus, although the mode and extent of fat storage in the liver have been shown (cf. Rapson *et al.* 1945) to be associated with wide differences in fatty acid composition, for such fish as the groper and the *Merluccius* species (van Rensburg *et al.* 1945) these factors have not been found important in the school shark. The present comparison of the New Zealand school shark with the similar if not identical Australian species, and with the ling, suggests that in some cases the species factor is of primary importance in determining the fatty acid composition of the liver oil.

It is quite possible that the factors which determine the fatty acid composition of the liver oils of

Table 8. *Comparison between the fatty acid composition of the liver oil of the school shark and of New Z*

Table 8. *Comparison between the fatty acid composition of the liver oil of the school shark and of New Zealand ling**

	Fatty acids (% w/w)										Liver oil expressed as percentage of total oil in fish	Oil in fish (%)
	Saturated					Unsaturated						
	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}	
New Zealand school shark: Mean value	2·3	16·1	4·7	0·7	0·2	0·9 (−2·0 H)	5·7 (−2·0 H)	27·6 (−2·3 to −2·6 H)	19·0 (−4·5 to −5·8 H)	22·3 (−6·5 to −9·6 H)	0·5 (−4·0 H)	69–89	1·3–3·8
Standard error (4 samples)	±0·55	±0·45	±0·75	±0·40	±0·25	±0·15	±0·20	±1·4	±1·1	±1·3	±0·55		
Australian school shark	2·6	18·7	5·1	0·1	—	0·3 (−2·0 H)	8·4 (−2·0 H)	27·2 (−2·4 H)	17·8 (−6·6 H)	19·8 (−12·0 H)	—		
New Zealand ling (Shorland, 1939): Mean value	1·8	16·4	2·5	—	—	—	7·2 (−2·0 H)	36·1 (−2·1 to −2·6 H)	24·3 (−4·9 to −5·7 H)	11·7 (−6·9 to −9·7 H)	—	c. 99	2·9
Standard error (7 samples)	±0·17	±0·30	±0·31	—	—	—	±0·47	±0·64	±0·50	±0·85			

* Shorland (1939).

elasmobranchii differ considerably from those operating in teleostean species. As shown by Hilditch (1941) the former have a much greater range of variation in the nature of the unsaturated acids, which vary from predominantly monoethenoid types to types which are even more unsaturated than those normally encountered in teleostean species. These facts, together with other remarkable differences in the amount and nature of the unsaponifiable matter in the liver oils of elasmobranchii, tend further to emphasize the importance of the species factor in determining the fatty acid composition of this group. Lovern (1942) has classified elasmobranch liver oils into four main groups on the basis of the nature and amount of unsaponifiable matter and the fatty acid composition. Consideration of the school shark-liver oil shows that the higher unsaturated acids are largely polyethenoid, and the fatty acid composition is not very different from that of the average marine fish oil. In this respect the school shark is shown to conform to Lovern's first group from which, however, it must be distinguished by the presence of much more unsaponifiable matter (2·5–31·4%) as compared with usually not more than 1 or 2% considered typical of that group.

SUMMARY

1. Studies of the distribution of oil in eight specimens of school shark (*Galeorhinus australis*) showed that the liver, which varied in oil content from 23·1 to 60·7%, contained from 66·8 to 93·0% of the total oil reserves of the fish. Ester-fractionation analyses of four of the liver oils, and of the phosphatide and glyceride fractions of the combined head and body lipids, showed that the liver fatty acids contained a higher content of palmitic acid and of C_{18} unsaturated acids, but less stearic acid than the head and body lipids.

2. In regard to most groups of fatty acids the liver oils showed great similarity. The wider differences in content of C_{18}, C_{20} and C_{22} unsaturated acids, amounting to as much as 6·3 units % are thought to lie outside the experimental error, but variations in accuracy shown by the results of various investigators are such as not to preclude such differences being due to experimental error.

3. Consideration of the variations in the oil content as a factor in determining the fatty acid composition of *G. australis* liver oil suggest that this fish is not influenced appreciably in this way, and thereby presents a striking contrast to the teleostean species studied by Rapson and collaborators.

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Studies on Suramin (Antrypol, Bayer 205)

6. FURTHER OBSERVATIONS ON THE DETERMINATION OF SURAMIN IN WHOLE BLOOD AND SERUM

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The amount of the trypanocidal drug suramin present in the blood serum or plasma of man, and any other animal which has received an injection of the drug, can readily be determined by a method previously described (Dangerfield, Gaunt & Wormall, 1938; Boursnell, Dangerfield & Wormall, 1939). The technique of this determination has deliberately been made as simple as possible in order that the method might be of use in tropical countries, even in regions where the simplest laboratory facilities are not available. The possibility has always been kept in mind that medical officers in regions well removed from the central hospitals might require blood suramin determinations on some of their treated trypanosomiasis patients. The drug has a very definite prophylactic value, and plasma suramin determinations might be made in future on large groups of people who have been injected with the drug before being exposed to infection.

Serum and plasma from suramin-containing blood contain the same concentration of suramin, and either material serves for the determination of the amount of the drug in the blood. Should the serum or plasma become grossly infected, however, the resulting bacterial changes might produce amines and other compounds which would vitiate the results. Experiments have, therefore, been carried out to determine whether any simple antiseptic can be added to the serum or plasma to preserve it for subsequent suramin determinations without interfering with the colorimetric determination. Experiments have also been carried out to investigate the reliability of suramin determinations on whole blood samples. Although these investigations were primarily designed for the above-mentioned purposes, they have also furnished useful information about the distribution of the blood suramin between the cells and the plasma.

METHODS

Suramin determinations. These were carried out as described previously (Dangerfield *et al.* 1938; Boursnell *et al.* 1939). The volume of each sample was made up to 3·0 ml. with distilled water before the acid hydrolysis. Blank determinations were made on blood serum or plasma to which no suramin had been added, and these control solutions were placed in the 'backing' tube of the comparator to compensate for a small amount of 'non-suramin' amine precursor in blood samples.

Some commercial samples of suramin when hydrolyzed, diazotized, and coupled with methyl-α-naphthylamine, gave a slightly stronger pink colour than did the sample used originally for the preparation of the Tintometer discs, i.e. they gave a 'recovery' of 101–108%, but this difficulty was readily overcome by standardizing each fresh batch of suramin. These slight differences between different suramin preparations might be partly due to differences in their water content (see also Hawking, 1941). Since, however, all our samples were dried *in vacuo* over $CaCl_2$, it seems more probable that there is a slight variation in the composition of commercial suramin.

RESULTS

The recovery of suramin added to whole blood

Varying amounts of suramin were added to defibrinated or oxalated blood (ox, rabbit and human) and determinations were carried out on the whole blood, and in some cases on the separated cells and plasma. The results of typical experiments (Table 1) show that the recovery of the added drug is low and very variable. Duplicate determinations usually gave good agreement, but the percentage recovery with any one sample of blood varied with the amount of suramin added. The latter observation excludes the use of a 'recovery' factor for calculating the true suramin content of whole blood.

It was thought possible that the recovery of suramin might be improved if the acid hydrolysis of the whole blood sample were more vigorous, e.g. by the use of a larger volume of HCl or by hydrolyzing for 9 hr. instead of the customary 6 hr. Where 5 ml. of HCl were used, the recovery (69–97%) was higher than that obtained with 3 ml., but the difference was not sufficiently marked to justify this change of technique. An increase in the duration of acid hydrolysis led to slight or negligible increases in the recovery of suramin added to whole blood, and more prolonged acid hydrolysis is not desirable since it has been found that further heating beyond the normal 6 hr. period leads to loss of the hydrolyzed suramin (Dangerfield *et al.* 1938).

The recovery of suramin added to washed red blood cell suspensions. Since there is approximately complete recovery of suramin added to serum or plasma, it was concluded that the lower recovery with whole blood samples was due to constituents of the red cells, or more probably to products produced by acid hydrolysis of these cells. Suramin determinations have therefore been made on mixtures of suramin and suspensions of washed red blood cells (ox and human); again the recovery was very low.

Possible reasons for the low recovery of suramin added to whole blood. The low recovery of suramin from whole blood samples is apparently due to some constituent of the red cell or a product formed by

Table 1. *Recovery of suramin added to whole blood*

(Suramin was dissolved in 0·9% (w/v) NaCl and 1·0 ml. of the solution was added to fresh defibrinated ox or human blood or oxalated rabbit blood.)

			Acid hydrolysis		
Type of blood	Volume of blood (ml.)	Suramin added (mg.)	Amount of 10·3 N-HCl (ml.)	Duration (hr.)	Recovery* (%)
Ox	0·50	0·44	3·0	6	86
			3·0	9	86
			5·0†	6	97
	2·0	0·44	3·0	6	69
			3·0	9	75
			5·0†	6	83
Rabbit	2·0	0·50	3·0	6	52
			5·0†	6	69
	2·0	2·0	3·0	6	60
			5·0†	6	76
Human	2·0	1·03	3·0	6	66
			5·0†	6	76

* In this and subsequent tables the results recorded are average values for two duplicate determinations.

† When 5·0 ml. of 10·3 N-HCl were used, the hydrolyzed solution was diluted with distilled water to 16·7 ml. (as against 10·0 ml. when 3·0 ml. of HCl were used) to give the right acidity (approx. 3 N) for satisfactory diazotization and coupling.

hydrolysis of the cells, which inhibits the diazotization or coupling, or which reacts with the amines derived from suramin. Experiments were therefore carried out to determine the recovery of suramin after its addition to HCl-hydrolyzed blood or red blood cells. The results showed that quantitative recovery was obtained under these conditions. The loss appears to occur, therefore, when the suramin is heated with HCl in the presence of red cells.

The preservation of serum for suramin determinations

Many experiments have been carried out with a variety of commonly used antiseptics to determine which are satisfactory for the preservation of serum for subsequent suramin determinations. The following antiseptics were added to serum from rabbits injected with suramin, or to normal human serum to which suramin had been added: ether, chloroform and toluene (0·3, 0·2 and 0·2 ml. respectively/2 ml. of serum); phenol and merthiolate (to give concentrations of 0·2–0·4 and 0·018 % respectively); 10·3N-HCl (3 ml./2 ml. serum). Suramin determinations made on these treated sera after they had been standing at 17–25° for periods of 1–5 weeks showed that ether, chloroform and toluene are not completely satisfactory for preservation; the results usually agreed with determinations made before the preservation of the serum, but occasionally low results were obtained. The HCl-treated samples were sometimes

Table 2. *Distribution of suramin added to whole blood*

Mixture	Defibrinated ox blood (ml.)	Suramin added in 1·0 ml. of 0·9 % NaCl (mg.)	Suramin found (mg./21 ml. of the mixture, or/fraction obtained from 21 ml. of the mixture)		
			Whole blood	Cells*	Plasma
A	20	20·0	12·8	1·4	17·0
B	20	3·5	2·4	0·2	3·2

* For reasons given in the text the cells were not washed, and the small amount of suramin found in them was probably present in the plasma adhering to the cells.

Distribution of added suramin between the red cells and plasma of whole blood

As a preliminary to an investigation on the distribution of suramin between the plasma and cells of the blood of animals injected with the drug, experiments have been carried out to determine how suramin added to the whole blood is distributed. Suramin (dissolved in a little 0·9 % NaCl) was added to fresh defibrinated ox blood, and samples of the well shaken mixture were centrifuged and the plasma and red cells separated as completely as possible. The cells were not washed in this experiment, in order to avoid the loss of any suramin which might be loosely combined with the cell membrane, or any which might diffuse through the cell wall during the washing. The results (Table 2) show that the added suramin became associated completely or almost completely with the plasma. The small amount present in the red cell fraction in this experiment can almost certainly be accounted for by the small amount of plasma remaining in the unwashed red cell fraction.

The recovery of the suramin from the whole-blood mixtures (64 % for A and 69 % for B) was similar to that obtained in other comparable experiments. The total recovery from the separated plasma and red cells, i.e. the sum of the values for the two fractions, was quite satisfactory (92 and 97 %), the slight loss being due to incomplete recovery of the small amount of the drug present in the red cell fractions.

In other experiments, suramin was added to suspensions of red blood cells to determine whether any of the drug became attached to the cells either immediately or when the mixtures were allowed to stand, with gentle shaking, for 3 hr. The results of these experiments, some of which are given in Table 3, show that no significant amount of the added drug became attached to the red cells.

Table 3. *Addition of suramin to suspensions of washed red blood cells*

(Fresh defibrinated blood was centrifuged and the red cells washed several times with 0·9 % NaCl solution, and then suspended in sufficient 0·9 % NaCl to give a volume equal to that of the original blood sample.

Mixture A: 20·0 ml. of ox red blood cell suspension + 22·1 mg. of suramin in 1 ml. of 0·9 % NaCl.

Mixtures B and C: 20·0 ml. of human red blood cell suspension + 21·6 mg. of suramin in 1 ml. of 0·9 % NaCl.

The mixtures were kept at 21° for 15 min. (A and B) or 3 hr. (C) with occasional gentle shaking, and 5 ml. samples of each were then centrifuged. The cells were washed three times with 0·9 % NaCl (3 ml. each time), and the washed cells suspended in 0·9 % NaCl to give a final vol. of 5·0 ml. The supernatant solution and washings from each sample were combined, and 0·9 % NaCl was added to give 20·0 ml.)

Mixture	Suramin added (mg.)	Suramin found (mg./21 ml. of A, B or C, or/fraction obtained from 21 ml.)		
		Original cell suspension	Washed red cells	Supernatant plus washings
A	22·1	17·0	0·0	22·1
B	21·6	15·8	0·2	21·0
C	21·6	16·4	0·2	20·8

satisfactory, as was noted by Hawking (1940*a*), but they occasionally gave surprisingly low results; no explanations for this can be offered at present. The results with the sera preserved with phenol or merthiolate were invariably satisfactory. Serum samples to which no antiseptic had been added became grossly infected when kept at room temperature for 1–5 weeks, and they often gave high suramin values.

DISCUSSION

Determinations of the amount of suramin in the blood can yield useful information about the prophylactic value of the drug, and about the blood level which might reasonably be expected to confer immunity against trypanosomiasis. Vierthaler & Boselli (1939), for example, found that a concentration of 1·3 mg. of the drug/100 ml. of plasma, as determined by the method developed in our laboratory (Boursnell *et al.* 1939; Dangerfield *et al.* 1938), was sufficient to protect rabbits against infection when injected with *Trypanosoma brucei*. Hawking (1940 *a*, *b*) concluded that defective accumulation of the drug in the blood was one of the main factors responsible for relapses in three patients treated with suramin for African sleeping sickness; he also found that numerous living trypanosomes reappeared in the blood of one patient even though the plasma contained 4·5 mg. of suramin/100 ml.

It is possible that investigators in tropical countries might wish to make suramin determinations, by our method, on samples of whole blood, but the low results obtained in our experiments indicate that plasma or serum must be used to obtain a true picture of the blood suramin level. Where samples of sera or plasma have to be kept for some considerable time before suramin determinations can be carried out, it is advisable that some antiseptic should be added; gross infection sometimes produces appreciable amounts of amines, or amine precursors, which cause a high result to be obtained in the suramin determination. Of the limited number of antiseptics tested by us, phenol and merthiolate have proved most satisfactory, and it is recommended that 0·2 ml. of 5% (w/v) phenol or 0·1 ml. of 0·5% (w/v) merthiolate should be added to 2 or 3 ml. of plasma or serum. The use of phenol for this purpose might be specially recommended, since it has been shown (Wormall, 1933) that phenol-treated serum can be used for the diagnostic red cell adhesion test for human trypanosomiasis (Duke & Wallace, 1930; Wallace & Wormall, 1931).

The long retention of injected suramin in the animal body can be attributed to the combination of the drug with the plasma and tissue proteins. In view of the ready combination of the drug with many proteins (for the literature see Boursnell & Wormall, 1939; Dewey & Wormall, 1946; Spinks, 1948), it seems rather surprising that no significant amount of suramin combines with the red blood cells. In our experiments, red blood cells took up no suramin when they were mixed with suramin solutions and left for up to 3 hr., and, in experiments which will be reported later, little suramin has been found in the red cells of the blood of rabbits injected with the drug.

SUMMARY

1. Suramin determinations made on whole blood samples, by the method previously found satisfactory for serum and plasma, show a low recovery of the suramin.

2. This low recovery is due to constituents of the blood cells.

3. Suramin added to whole blood is almost completely confined to the plasma, and none combines with the red cells.

4. The addition of phenol or merthiolate to serum samples is recommended if these samples have to be kept for some time before suramin determinations are made.

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The Fractionation of Potato Starch

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The fractionation of starch by precipitation with butanol and similar alcohols, first developed by Schoch (1942), stimulated research in this field by providing the first efficient method of preparing in reasonably pure form the components, amylose and amylopectin. These names have often been applied to the corresponding fractions obtained by earlier methods in various degrees of purity, and in consequence are associated in the literature with many conflicting statements. They are nevertheless retained here, it being understood that amylose, consisting essentially, if not solely, of unbranched chains of α-glucopyranose units, is the fraction precipitated from starch paste by butanol, amylopectin remaining in dispersion.

It is supposed that amylose is completely hydrolyzed to maltose by β-amylase, although the evidence for this is not entirely satisfactory, since pure substrate and enzyme have rarely, if ever, been employed in the same experiment. Tests of the purity of starch fractions include the limit of β-amylase action, analysis of iodine coloration and potentiometric titration with iodine; the first two have been extensively employed in this work. In the present investigation, attempts have been made to obtain pure amylose and amylopectin by the known precipitation methods, and if possible to improve upon these methods.

Schoch (1942) autoclaved starch paste with butanol or *iso*amyl alcohol, and later (Schoch, 1945), after autoclaving alone, added excess of pentasol, a commercial mixture of primary amyl alcohols. The liquid was cooled very slowly, amylose coming out in crystalline needles or rosettes which were separated by high-speed centrifugation. It was purified by recrystallization from the same solvent mixture. Amylopectin was precipitated from the mother liquor with methanol. Autoclaving is considered by Schoch (1945) to be essential to the success of the fractionation; otherwise, the fractions are markedly contaminated with one another.

Haworth, Peat & Sagrott (1946) precipitated the amylose from non-autoclaved starch paste in the cold with thymol. The avoidance of autoclaving is a valuable feature of this method, since mild hydrolytic decomposition of amylose and amylopectin, even in 2 hr. at 120° at pH 6 as employed by Schoch, may be feared. These authors also suggested the use of the *cyclo*hexanols as precipitants, and Whistler & Hilbert (1945) that of the nitroparaffins.

EXPERIMENTAL

Materials. Commercial samples of potato starch, the best obtainable, were employed. One sample gave a paste of pH 4·5. By washing the starch three times with 0·001 N-K_2CO_3 followed by distilled water, the reaction of the paste was changed to pH 5·8–6 as required by Schoch (1942). This starch yielded about 17% of purified amylose. Another sample, for example, yielded 20% amylose and a less viscous starch paste.

Starch was mixed to a cream with cold water, and added to boiling water with vigorous stirring. After gentle boiling for 30 min. to yield a homogeneous paste, the latter was either (1) treated with 0·05% NaCl and rapidly cooled, or (2) heated in an autoclave at 18–20 lb. pressure for 2 hr. as described by Schoch, the NaCl being omitted. The pastes contained about 1·5% of starch.

Determinations of yields of fractions. A weighed sample of a solution was evaporated to dryness, dried at 104° and allowance made for any non-carbohydrate constituent if present.

Determination of blue value. The procedure of McCready & Hassid (1943) was followed with slight modification, but different units were employed. A solution containing 1 mg. of carbohydrate/100 ml. and 0·002% of I_2 in 0·02% KI was read in a 1 cm. cell in a Spekker absorptiometer against iodine, using (unless otherwise stated) the 608 filter (6500–7000 A. transmission), which corresponds closely to that employed by McCready & Hassid. The reading under these conditions is the blue value. In some investigations, other filters, e.g. 607 (5900–6300 A.), were also employed (see text).

Fractionation procedure

Preliminary experiments yielded 23% of crude and 17% of fully purified amylose from one potato starch by Schoch's (1942) method, using butanol. Purification was achieved by redissolving the crude amylose to 0·7–1·0% concentration in hot water saturated with butanol, and cooling from 80° or 90° over a period of 3–5 hr. The precipitate was washed with butanol-saturated water until the washings no longer gave any colour reaction with I_2, redissolved at 80°, reprecipitated and washed. Three reprecipitations, involving nine washings, yielded a product from which no more amylopectin could be extracted, as indicated by the absence of iodine coloration or dissolved carbohydrate in the mother liquor.

Amylose precipitated by thymol (Haworth *et al.* 1946), or *cyclo*hexanol followed by thymol, was separated as soon as possible by centrifuging, redissolved in hot butanol-saturated water, reprecipitated and washed several times as described above.

The original mother liquor was concentrated *in vacuo* and the amylopectin precipitated with ethanol. Foaming created difficulties when thymol had been used, and the evaporation was sometimes omitted.

The best amylopectin was obtained as follows: To 1·5–2% starch paste (prepared with NaCl as in (1) above) *cyclo*hexanol was added, drop by drop with stirring, up to 0·15–0·2% of the paste. Stirring must not be continued until the paste becomes milky. After standing 3 days, 0·15% of powdered thymol was brought into suspension by gentle stirring. The amylose precipitated within 2 days and was separated by centrifuging. The amylopectin had a blue value of about 0·02, whereas that from the thymol precipitation alone was about 0·025–0·03. If stirring with *cyclo*hexanol increases the opalescence of the paste the resulting amylopectin is found to have an increased blue value.

Precipitation of amylose from starch paste by the method of Haworth *et al.* (1946), followed by rapid drying, gave a product which was only slightly soluble in water. The original centrifuged preparation could, however, be purified by recrystallization from butanol-saturated water, which was preferred to thymol water for this purpose. In effect, it was thereby converted into the more stable butanol complex, and it was usually stored in this form without drying.

There appears to be no necessity to heat the starch paste under pressure before precipitating the amylose, even if butanol is used. Two preparations of amylose were carried out by butanol precipitation, the one from paste autoclaved for 2 hr. at 120°, the other from paste which never rose above 90° at any time. The latter gave some trouble, but the redissolved amylose was successfully separated from remnants of burst starch granules, which could not be easily removed from the original paste. The two amylose preparations were readily soluble, and gave similar blue values (0·33), but that from autoclaved paste was hydrolyzed by β-amylase much the faster, each reaching the same limit. The corresponding amylopectins possessed identical blue values (0·024), and hydrolyzed at almost the same rates, the 120° preparation only slightly the faster.

So far as the amylose was concerned it did not greatly matter which precipitant was used, butanol, thymol or *cyclo*hexanol followed by thymol, provided that the separated complex was promptly washed and recrystallized from butanol water. In one instance, using the same non-autoclaved starch paste, butanol and thymol precipitations were performed side by side. The butanol amylose, recrystallized three times, gave a 17·2% yield, blue value (B.V.) 0·355. Thymol-precipitated amylose, recrystallized three times from butanol water, gave a 17·7% yield, B.V. 0·345. The light absorptions of the respective blue liquids were almost identical from 4300 to 5800 A. However, the thymol preparation gave the lower absorption from 5800 to 7000 A. Under the same conditions, both amyloses hydrolyzed at the same rate with β-amylase, and reached the limit of 99·5% of theoretically obtainable maltose.

The crude thymol-amylose was bulky and did not adhere to the walls of the vessel, but after recrystallization from butanol water it occupied only 40% as much space as before and adhered to the walls. It underwent no further change in these respects during further recrystallizations from butanol water. It was found unsatisfactory to leave it long in contact with thymol water as it lost solubility and affinity for I_2.

The butanol-amylose complex, on heating with water saturated with butanol, begins to disperse at 65° to form an opalescent solution which clears at about 73°. On cooling slowly the phenomenon of supercooling may be observed as far down as 35°.

Schoch (1942, 1945) stipulated that the reaction of potato starch paste must be about pH 5·9–6·3 for successful fractionation. In this work, there were indications that wider variations in pH were permissible; indeed, successful precipitations from non-autoclaved paste were effected even at pH 4·1 and 9. But certain differences in behaviour were noted in extreme cases. Thus, amylose precipitated from non-autoclaved paste by butanol, thymol or *cyclo*hexanol followed by thymol, brought by HCl addition to pH 4·1, was more easily washed and freed from amylopectin than in parallel precipitations at pH 6 and 9. It also showed a greater tendency to retrograde in neutral, hot aqueous solution, and when dried on a watch glass it was opaque. It was hydrolyzed by β-amylase in dilute solution faster than amylose precipitated at pH 6 or 9 hydrolyzed under identical conditions.

No significant differences were noted in the amylopectins except that the lowest blue values were obtained in the *cyclo*hexanol + thymol method (Table 2).

RESULTS

General properties of amylose

Amylose forms a viscous solution when concentrated, which may cool to a gelatinous consistency. Solutions of over 0·5% retrograde fairly readily, and this has been a source of difficulty in previous work. Whenever amylose approaching a high degree of purity has been prepared in these laboratories, e.g. by electrophoresis, retrogradation has been liable to occur, rendering the product permanently insoluble in water and useless for many purposes. Precipitates from hot solutions on cooling may not be retrograded material, but may redissolve with more hot water. Retrogradation may be avoided by high temperature, low concentration, maintenance of neutral pH, and over long periods by preservation in the form of a complex with butanol, thymol and nitroparaffins. Recrystallization from butanol water need not involve a cooling period greater than 3–5 hr. In the form of the butanol complex, i.e. in the presence of butanol-saturated water, even the purest amylose is stable for months and shows no signs of retrogradation. The supernatant liquid in such a case contains less than 1 mg. solid matter/100 ml. and gives only a yellow colour with iodine.

Criteria of purity of amylose

The distinction between the iodine colorations of amylose and amylopectin consists essentially in the intensity of the blue. While amylopectin shows no great variation from 5000 to 8000 A., amylose exhibits a marked minimum transmission near 6000 A. Kerr (1944) places this at 6000–6200 A. for potato amylose which corresponds to light filter 607 as

used with the Spekker absorptiometer. For different preparations we have found from 5800 to 6100 A., the highest reading on the absorptiometer being obtained with the 607 filter. After making allowance for variation in experimental error when using these filters, the one should be chosen which gives the greatest numerical difference between amylose and amylopectin. This is when the need is to characterize different preparations of amylose or to measure amylose present as an impurity in amylopectin. For the reverse purpose, that of measuring blue value of both the amylopectin which remains contaminated with it and the amylose which has lost it. A perfectly representative sample of amylose cannot be obtained unless the first precipitation is performed in the right way.

The blue value was found to be unaffected by small quantities of butanol and maltose. It is chiefly influenced by the temperature at the time of reading, and by the conditions under which the amylose has been crystallized from solution, and, generally, the amount of exposure it has had to hot

Table 1. *Blue values of amylose after various periods of heating and cooling*

	Light filter (no.)	Blue values: Original sample	Original sample dissolved, heated, saturated with butanol and cooled: From 70° in 3·5 hr.	From 90° in 3·5 hr.	From 90° in 1·5 hr.
Solution in cold water	608	0·348	0·318	0·336	0·352
	607	0·361	0·335	0·350	0·369
	606	0·292	0·285	0·290	0·298
Solution held at 90° for 10 min. before testing	608	0·339	0·316	0·330	0·350
	607	0·356	0·335	0·346	0·364
	606	0·291	0·277	0·287	0·296
Solution held at 90° for 30 min. before testing	608	—	0·302	0·330	0·316
	607	—	0·332	0·348	0·335
	606	—	0·272	0·287	0·277

Filter 608, 6500–7000 A.; 607, 5900–6300 A.; 606, 5700–5900 A.

amylopectin or red iodine complexes in amylose, the filter selected should transmit light of about 4800–5000 A., the 603 filter being indicated.

Failure to remove amylopectin or other carbohydrate by recrystallization from cooling butanol-water solution is an indispensable criterion of purity. The process should be accompanied by no change in blue value (so long as recrystallization is brought about under the same conditions and is complete). Short of adequate investigation of molecular structure, perhaps the best further criterion available to us is the completeness of hydrolysis by pure β-amylase together with some kinetic features of this hydrolysis which are dealt with in a separate communication.

The blue value is of use during the process of freeing amylose from adherent amylopectin, and its value rises to a maximum during successive recrystallizations. But the value may be different in one preparation from that of another and seems to depend both on the initial conditions and on those of the final recrystallization.

Some amylose is normally left behind in the amylopectin, probably relatively low molecular amylose, which is difficult to remove. That fraction of the native amylose which is not precipitated in the first instance will vary from process to process, and from precipitant to precipitant, and will influence the water. Table 1 shows a series of values (measured with three filters) of one specimen dissolved and recrystallized under different conditions. The products (butanol complex) were each dissolved in three ways. These results mainly serve to show that, within the range of the experimental conditions, the slower the cooling the lower the blue value. A smaller influence is exerted by conditions of dissolving, standing at 90° lowering the value. Evidently, these conditions must be controlled if the blue value is to be used as a criterion.

From this and other experiments it appears that when amylose is unduly exposed to hot water a part may precipitate, this being irreversibly insoluble (retrogradation), whilst more may be changed but remain in solution. The solution now exhibits a lower blue value than before and can no longer be hydrolyzed to 100 % of maltose by β-amylase. The same is true of amylose which has been precipitated and washed by ethanol and ether. It is less soluble in water and the soluble portion has a lower blue value than before this treatment. This changed, soluble amylose is apparently related to the natural amylose much as denatured protein is to the corresponding native form. On the other hand, retrograded material would seem to correspond to protein which has been both denatured and coagulated. The blue value is not related to the mean chain

length of the molecules, but to the extent of denaturation. As amylose is purified it shows an increasing tendency to retrograde.

Recrystallization from hot aqueous solution by half saturation with butanol effects no fractionation of amylose so far as is indicated by blue values, provided that two conditions are fulfilled. The precipitated portion and that remaining in solution must each be separately recrystallized from hot butanol-saturated water and redissolved under the same conditions, and there must be no denaturation. If there are any signs of retrogradation, such as marked opalescence, the unprecipitated fraction is found to have a lower blue value than the precipitate, e.g. 0·310 as against 0·340.

Purification and properties of amylopectin

Amylopectin was not further purified or its blue value lowered by reprecipitation by thymol, butanol, acetone, chloroform, or thymol-*cyclo*hexanol mixture in equal parts, by shaking to facilitate retrogradation of amylose or by filtration through cotton. The latter adsorbed added amylose, however. The blue value was not altered from that of the first amylopectin precipitate, although this value varied markedly according to the original method of precipitating the amylose. Thus, purer amylopectin was obtained by precipitation with thymol than with butanol, and still better with *cyclo*hexanol followed by thymol.

Presumably, the amylose remaining entangled with the amylopectin consists of short molecular chains which the precipitant fails to pick out. Various precipitation procedures were tried. For example, ethanol was added to 1–2 % of amylopectin in 0·05 % NaCl until a cotton-like precipitate (I) was formed. This was separated and the mother liquor allowed to stand. In the course of the following days a further precipitate slowly formed (ppt. II). The results in Table 2 suggest that a little contaminated amylose was preferentially removed with the first precipitate, but neither preparation is considered to be entirely free from amylose. β-Amylase indicated no difference between the first and second precipitates, which hydrolyzed at the same rates and reached the same limits.

Amylopectin is in general hygroscopic. It adheres so strongly to glass or porcelain that in the course of drying the shrinking material picks particles of glass from the surface of the vessel. This is specially noticeable on cooling the dried amylopectin, the differences in thermal expansion of glass and amylopectin coming into operation.

SUMMARY

1. Amylose and amylopectin have been prepared from non-autoclaved potato starch paste by successive additions on alternate days of *cyclo*hexanol and thymol, which, on the whole, gave the best results, and by the methods of Schoch and of Haworth, Peat & Sagrott.

2. No great differences were noted among the products obtained by use of three precipitants.

3. There is no advantage to be gained by heating the starch paste under pressure. If this is omitted a fairly wide range of pH may be tolerated at the time of precipitation, but the amylose produced under acid conditions showed the greater tendency to retrograde in neutral solution.

4. Preparations of amylose with 17 times the blue value, as measured on the Spekker absorptiometer, of the corresponding amylopectin have been obtained.

5. The blue value of amylose is greatly influenced by the method of recrystallization from butanol-saturated water, and by the period of heating when redissolving.

Table 2. *Blue values of amylose and amylopectin*

		Blue values			
			Amylopectin		
				Ethanol precipitates	
Precipitant	pH at time of precipitation	Amylose	Crude	I	II
Butanol	4·1	0·285	—	0·028	0·025
	6·0	0·310	0·024	—	—
	9·4	0·320	—	0·027	0·025
Thymol	4·1	0·302	—	0·029	0·025
	6·0	0·332	—	—	—
	9·4	0·342	—	0·028	0·025
*cyclo*Hexanol followed by thymol	4·1	0·318	—	0·028	0·022
	6·0	0·336	0·021	—	—
	9·4	0·334	—	0·027	0·023

6. Exposure of amylose to hot water brings about changes even in the portion which does not retrograde. The blue value falls and β-amylase action is incomplete.

7. Amylopectin was not so successfully purified, but fractional precipitation with ethanol freed it slightly from amylose which had escaped the first precipitation.

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The Action of β-Amylase on Potato Amylose

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It has long been known that almost all natural starches contain a constituent which can be hydrolyzed to completion by β-amylase. It has been given various names, that of amylose being the one used here. It is only in quite recent years that amylose has been prepared in a state of purity (Schoch, 1942; Haworth, Peat & Sagrott, 1946). β-Amylase has usually been reported as promoting the hydrolysis of amylose to 90–100% of maltose, but many of these figures may be above the truth. Often, it has not been made clear whether the figure reported referred to maltose expressed as a percentage of the moisture-free starch used or of the maltose theoretically obtainable from it. The absence of glucose from the products has been assumed, although many preparations of β-amylase contain maltase. Lastly, it is rare that the β-amylase has been demonstrated to be free from α-amylase and from maltase.

In this work, the purest amylose and β-amylases obtainable have been investigated with special attention to the kinetics of the reaction and a hypothesis is advanced to account for the facts.

MATERIALS AND METHODS

Substrates. The various preparations of amylose are described elsewhere (Hopkins & Jelinek, 1948). Solutions not exceeding 0·9% concentration were prepared by heating the butanol-amylose complex in water until the butanol was driven off.

Blue value (B.V.). The procedure of McCready & Hassid (1943) as modified by Hopkins & Jelinek (1948) was employed.

Red value (R.V.). This was measured in the same way as the B.V., except that filters 602 and 603 were used with the Spekker absorptiometer. It was found of advantage, however, to increase the iodine concentration from 0·002 to 0·008%.

Enzyme preparations. β-Amylase was prepared (1) from well-ripened barley as described by Hopkins, Murray & Lockwood (1946), (2) from soya beans by the method of Newton & Naylor (1939) and Newton, Hixon & Naylor (1943). Both specimens were tested and found to be free from maltase activity under the appropriate conditions of use. The limits of hydrolysis of starch pastes were 57% of the theoretical maltose ($R_m = 57\%$), and the preparations were considered to be free from α-amylase.

Reaction mixtures. These were as employed by Hopkins *et al.* (1946), but contained varying concentrations of amylose as stated in the tables, and were buffered at pH 4·6 for barley and pH 5·8 for soya amylase. Progress of hydrolysis at 25° was determined as described by Hopkins *et al.* (1946), except that the hypoiodite titration was found not to be wholly satisfactory in the presence of certain antiseptics or if a trace of butanol was present. In such cases, the method of Cole (1933) was substituted, the ferricyanide being calibrated against maltose. Withdrawals from the reaction mixture were made into boiling water.

Calculation of the values of 'k' as if the reaction were monomolecular revealed that these decline progressively and markedly through the reaction. As is shown later, there was no theoretical reason for expecting monomolecularity.

For the determination of the affinity constant, a series of flasks was set up containing varying concentrations of the amylose (about four flasks for each concentration). All flasks contained identical concentrations of enzyme and buffer. After suitable intervals of time (estimated from a pilot experiment) pH 10 buffer was added to the respective flasks to check the reaction and the maltose determined by titration. The velocity of reaction v (mg. maltose/100 ml./min.) was obtained for each concentration of amylose substrate from the initial slope of the progress curves.

RESULTS

The limit of action was usually 99 % R_m, the full 100 % being obtained in some cases. An example is given in Table 1. The final reaction mixture from this experiment was concentrated after distilling off traces of antiseptics. The reducing power in a sample was determined by hypoiodite titration. After making allowance for weight and reducing power of the enzyme, the results indicated pure maltose. $[\alpha]_D^{15°}$ was found to be +136°.

Table 1. *Action of β-amylase (soya) on potato amylose* (B.V. 0·332)

(Concentration of amylose in reaction mixture 0·41%.)

Reaction time (hr.)	% of theoretically obtainable maltose (R_m)	Reaction time (hr.)	% of theoretically obtainable maltose (R_m)
0·5	49·3	3·0	85·4
1·00	62·7	3·5	88·4
1·33	68·5	4·0	89·3
1·5	71·0	5·0	92·8
1·75	74·1	20	97·6
2·0	76·4	90	99·1
2·5	82·2	192	100·0

Amylose prepared from autoclaved starch paste (120° for 2 hr. at pH 7, Table 2, no. 1) underwent hydrolysis about 1·5 times as fast as that similarly obtained from paste prepared at 90° (no. 2), but each reached the same limit. It may be remarked that the amylopectins prepared at the same time underwent hydrolysis at substantially the same rate as one another.

The reaction of the starch paste at the time of crystallization of amylose influenced the rate at which the amylose subsequently became hydrolyzed at pH 5·8. Of the amyloses in Table 2, one series, nos. 3, 4 and 5, had been precipitated respectively at pH 4·1, 6·0 and 9·4 by butanol, whilst nos. 6 and 7 constituted a pair precipitated at pH 4·1 and 9·4 respectively by *cyclo*hexanol followed by thymol. The choice of precipitant proved to be relatively unimportant, but the amyloses precipitated at acid reaction underwent hydrolysis the most rapidly. Associated with their liability to retrograde, discussed elsewhere (Hopkins & Jelinek, 1948), they failed to reach as advanced a limit of hydrolysis as the other samples. No. 3 partly came out of solution in hot water on two occasions before enzyme action was commenced. The substrate remaining in solution was hydrolyzed to an even lower limit (no. 8) than the whole sample (no. 3). During heating, some had precipitated, but more had been changed ('denatured') into a form less susceptible to β-amylase. The purer, i.e. the more 'native' the amylose, the more completely it is hydrolyzed and the higher its blue value (Fig. 1).

The results in Tables 1–3, e.g. nos. 1 and 2 of

Table 2. *Action of β-amylase (soya) on potato amyloses*

(Only reaction mixtures of a given series as mentioned in the text received identical quantities of the enzyme.)

(Results as percentage of theoretically obtainable maltose.)

No. of amylose sample (see text)	1	2	3	4	5	6	7	8	9
Feature of its preparation	Paste heated at 120°	Paste heated at 90°	Precipitated at pH 4·1	Precipitated at pH 6	Precipitated at pH 9·4	Precipitated at pH 4·1	Precipitated at pH 9·4	No. 3 partly retrograded	—
Blue value	0·322	0·332	0·285	0·310	0·342	0·308	0·326	0·252	0·327
% Amylose in reaction mixture	0·61	0·45	0·58	0·59	0·58	0·25	0·26	0·42	0·40
Time									
10 min.	25·0	19·5	19·9	14·8	12·8	20·1	13·3	26·0	—
20 min.	44·4	29·3	—	24·2	—	37·0	22·4	—	—
30 min.	—	—	—	—	—	—	—	47·4	—
40 min.	52·8	37·9	53·2	37·2	32·5	54·0	37·2	—	—
60 min.	61·7	46·7	—	46·4	40·1	63·9	46·0	—	—
90 min.	69·4	55·6	—	55·1	47·6	—	—	—	—
120 min.	73·8	—	—	60·3	54·2	81·6	62·3	—	—
150 min.	—	64·7	—	—	—	—	—	—	13·4
180 min.	79·6	66·7	—	69·5	62·6	86·0	69·3	—	—
210 min.	81·3	72·0	—	—	—	—	—	—	—
240 min.	82·4	74·0	—	—	—	87·3	75·1	—	—
300 min.	83·1	77·4	—	76·0	72·0	88·3	82·2	78·0	25·7
360 min.	84·4	80·4	—	—	—	—	—	—	29·6
435 min.	—	—	—	—	—	—	—	—	33·8
465 min.	—	—	—	—	—	—	—	—	34·9
11 hr.	—	—	—	—	—	—	—	—	44·2
24 hr.	—	—	91·5	91·5	95·9	92·5	95·0	82·5	62·2
Limit	97·4	98·0	94·0	—	98·0	94·8	99·0	85·5	—

Table 3, and the curves *A* and *B* of Fig. 1 show that the rate of hydrolysis is never constant but declines steadily. Except in the case of the partly 'denatured' amylose samples (e.g. nos. 3 and 6 of Table 2) there is no sudden break in the curve if percentage maltose is plotted against time. This

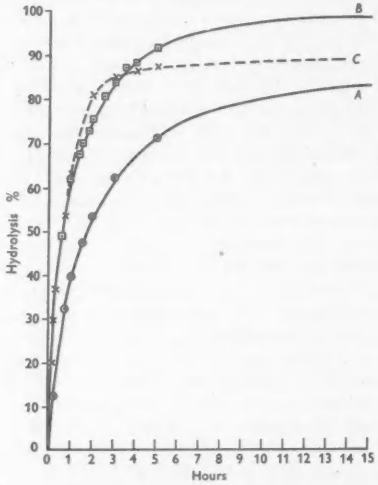

Fig. 1. Progress of hydrolysis of amylose by β-amylase (soya). The curves beyond 5 hr. were interpolated, making use of later readings which are not within the range of the graph. Curve *A*. From Table 2, no. 5. Limiting % 98. Curve *B*. From Table 1. Limiting % 100. Curve *C*. From Table 2, no. 6 (partly denatured amylose). Limiting % 95.

means that there is no evidence from our results of any branched or laminated chains in amylose, as has been suggested by other workers who observed such breaks. These do occur, however, with partly 'denatured' amylose samples, and the hydrolysis does not reach the full 100 % (Fig. 1, curve *C*).

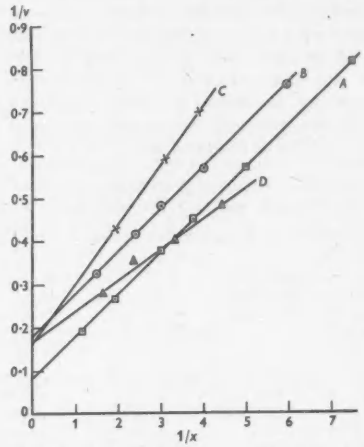

Fig. 2. Initial velocity-substrate concentration relationship plotted according to the procedure of Lineweaver & Burk (1934) for the system β-amylase (soya)-amylose. For amylose preparations *A–D* inclusive, see text.

	V (max. velocity) (mg. maltose produced/100 ml./min.)	K_m
A	12·0	1·17
B	5·45	0·53
C	6·25	0·86
D	6·05	0·45

Returning to the steady decline in velocity, this is more pronounced than if the reaction were monomolecular. At the same time the β-amylase-amylose

Table 3. *Action of β-amylase (barley) on potato amylose*

(Results as percentage of theoretically obtainable maltose.)

No. of sample (see text)	1	2	3
Feature of its preparation	Precipitated by thymol (not autoclaved)	Precipitated by butanol (not autoclaved)	Mixed samples
Blue value	0·345	0·355	0·336
% Amylose in reaction mixture	0·72	0·72	0·63
Time			
15 min.	7·5	—	22·2
30 min.	12·2	11·9	32·6
45 min.	16·4	—	—
60 min.	20·1	20·0	45·6
90 min.	26·3	—	53·2
120 min.	31·7	34·0	58·9
180 min.	—	44·4	66·8
240 min.	52·4	53·8	72·2
300 min.	—	61·4	75·8
360 min.	—	68·5	79·2
420 min.	—	74·2	—
480 min.	—	77·7	—
24 hr.	97·1	—	94·8
Limit	99·5	100·0	100·0

system complies with the Michaelis & Menten formulations in certain respects, since on plotting reciprocals of velocity ($1/v$) against those of concentration ($1/x$) straight lines were obtained (Fig. 2). The values of K_m (the affinity constant) and V (the maximum velocity attainable) were deduced by the usual procedure (Lineweaver & Burk, 1934). It is interesting to note that the affinity constants varied with specimens of amylose according to the conditions of precipitation. The 'amylose *A*' (Fig. 2) was precipitated from potato paste prepared at 100° by the addition of *cyclo*hexanol (0·15 %) and thymol (0·15 %) simultaneously at 25° following the suggestion of Haworth *et al.* (1946). Amylose separated in 3–5 days and was removed ('amylose *A*'). The residual paste was then heated to 100° and cooled slowly, more amylose separating ('amylose *B*'). Each amylose was purified as described by Hopkins & Jelinek (1948). The first fraction to precipitate (*A*) gave the higher value of K_m, but as K_m is expressed as a percentage and not as molarity (the mean molecular weight being unknown) this does not necessarily mean that this amylose possessed less affinity for β-amylase than the other. Evidence to be cited later indicates that the larger the amylose molecules the faster do they hydrolyze. This agrees with the high value of V for the first precipitate, the assumption being that it was the longer chain molecules which aggregated and came out of the paste the more readily.

Specimens *C* and *D* (Fig. 2) refer to two amylose 'fractions' prepared from one purified sample by partial saturation of its hot aqueous solution with butanol and slow cooling. Half of the amylose (*C*) separated from solution and was centrifuged off. The rest was recovered by full saturation (*D*). Again, the more precipitable fraction gave the higher value of K_m and of V. Evidently the numerical value of K_m for amylose and β-amylase varies widely according to conditions of preparation. It may bear a relationship to mean chain length. There are indications that in relatively concentrated solutions of amylose, v does not increase as predicted from the Michaelis formula. This is associated probably with 'denaturation' of the amylose under such conditions.

When amylose is undergoing hydrolysis with β-amylase the molecular chains are being progressively shortened. By withdrawing a portion of the reaction mixture, stopping the hydrolysis in it and recovering the residual polysaccharide by the butanol precipitation method, specimens were obtained of amylose in such stages of degradation. These in turn, when set up in identical reaction mixtures, were found to be hydrolyzed at widely different rates; the less degraded the amylose, the faster it underwent hydrolysis (Table 4). The partly hydrolyzed specimens were obtained by withdrawal from reaction mixture no. 9 of Table 2, *E* at 13·9 %, *F* at 44·2 % and *G* at 69 % (after 46 hr.). It seems clear that the further amylose is degraded by β-amylase, the more difficult it becomes for the amylase to hydrolyze it, assuming that no change in molecular character has occurred during the recovery of the partly hydrolyzed fractions.

Table 4. *Action of β-amylase on amylose, previously partly degraded*

(Results as percentage of theoretically obtainable maltose.)

No. of sample (see text) ...	Original	*E*	*F*	*G*
Blue value ...	0·327	0·335	0·327	0·339
% Substrate in reaction mixture ...	0·324	0·322	0·330	0·316
Time				
12 min.	24·0	—	20·6	15·6
22 min.	—	37·7	30·0	21·8
44 min.	58·5	51·6	44·4	31·7
60 min.	—	57·7	47·6	32·6
90 min.	—	62·0	—	—
150 min.	—	73·5	60·9	44·9
210 min.	—	79·2	69·6	51·0
300 min.	—	85·9	75·5	55·2
20 hr.	91·8	95·5	89·9	85·3

For the experiments reported in Table 5 enzyme action was checked by withdrawing samples and running into alkali instead of boiling water, since boiling may change the blue value of amylose. The percentage hydrolysis was determined by hypoiodite, and, after calculation of the concentration of residual polysaccharide, the liquid was diluted and used for the measurement of blue and red values. It was ascertained that these values were not influenced by the presence of maltose or the enzyme preparation. Samples *H* and *K* were prepared from amylose in the same way as *B* and *A* respectively of Fig. 2. Sample *H* was a normal sample, whilst *K* had been very slightly 'denatured' as shown by its behaviour.

Filters 602 (4300–4700 A. transmission) and 603 (4700–5100 A.) may be regarded as transmitting light which will be absorbed by red liquids. Readings in the Spekker absorptiometer using these filters are therefore 'red values' and will be relatively high in the presence of amylopectin or of dextrins of chain length about 7–12 glucose units. On the other

hand, with filter 607 (5900–6300 A.) scarcely any absorption by red solutions is measured, and with filter 608 none at all. With the latter filters, the absorption by amylose of greater mean chain length than 12 is measured. Only the values with filters 603 and 608 are recorded, but in several experiments a close parallel was observed between the 602 and 603 readings and also between the 607 and 608 readings.

Table 5. *Changes in blue and red values of residual polysaccharide during hydrolysis*

(Hydrolysis in percentage of theoretically obtainable maltose.)

Sample ...	*H* (normal sample)				*K* (slightly 'denatured')			
% Amylose in reaction mixture ...	0·64				0·62			
Time (min.)	Hydrolysis	Blue value (filter 608)	Red value (filter 603)	Ratio 608/603	Hydrolysis	Blue value (filter 608)	Red value (filter 603)	Ratio 608/603
0	0	0·346	0·131	2·64	0	0·327	0·127	2·56
40	56·0	0·327	0·130	2·52	53·4	0·282	0·117	2·41
70	66·5	0·310	0·129	2·40	61·1	0·258	0·107	2·41
100	74·3	—	—	—	67·6	0·247	0·102	2·42
130	78·6	0·280	0·120	2·33	72·2	0·230	0·100	2·30
160	82·0	0·255	0·114	2·24	75·2	0·199	0·090	2·21
240	88·6	0·220	0·109	2·02	81·9	0·164	0·077	2·13
300	92·2	0·179	0·100	1·79	86·0	—	0·066	—
360	93·8	0·128	0·084	1·53	88·0	0·084	0·048	1·75
420	95·0	0·078	0·069	1·13	89·8	0·040	0·039	1·03
500	96·9	0·059	0·076	0·77	90·7	0·021	0·032	0·66

DISCUSSION

In explanation of the various observations reported above the following hypothesis is advanced. Amylose consists of a mixture of chain molecules of various lengths, i.e. of a homologous series, the majority, according to the law of distribution, being of intermediate length. All but the relatively very short ones possess approximately the same blue value. Fractional precipitation, both from starch paste and from purified amylose, brings out the longer chains first, the shorter needing more drastic conditions to pick them out from the amylopectin, or to aggregate and crystallize them from aqueous solution. Heating changes the coiled pattern of the native macromolecules and renders them less blue in presence of iodine, less soluble in water and less completely hydrolyzable by β-amylase. With prolonged heating irreversible retrogradation occurs.

The longer chains are attacked preferentially and the more rapidly by β-amylase. The action of β-amylase is in one respect different from most enzymic reactions in which a single substance is decomposed. Successive maltose molecules are formed by the removal of two glucose groups at a time from the non-reducing end of the chain, a molecule of polysaccharide and a molecule of maltose being formed in each individual reaction. The more usual kinetics of an enzyme reaction cannot be expected since the formation of the reaction product, maltose, is not accompanied by a corresponding diminution of the molar concentration of the substrate. The percentage concentration of the latter diminishes, yet the molar concentration remains unaltered until one or more chains in the system become too short for further hydrolysis, either in the form of maltose or a trisaccharide. If all amylose molecules were attacked with equal readiness, irrespective of chain length, a zero-order reaction would be expected, whatever the concentrations of substrate and enzyme employed. This would persist until the shortest chains were exhausted and, unless the substrate was suitably dilute, until inhibition by reaction products or inactivation of the enzyme interfered. A zero order reaction, however, is never observed, not even with the highest concentrations of substrate employed.

On the other hand, if we assume that the longer the chain the faster it is hydrolyzed, we should expect a steadily declining rate of reaction, as is invariably observed. It could not be of zero order over any appreciable percentage of hydrolysis. But there is no reason to suppose that it would be monomolecular. With low concentrations of substrate and fast reactions the value of k should actually fall, as was usually observed. A consideration of the results in Table 4 will make this clear. Amylose *G* must have possessed a mean chain length of about one third of that of *E*, since it had previously been hydrolyzed by β-amylase to 69 %, compared with 13 % for *E*. As each was now freshly hydrolyzed at the same percentage concentration, the molarity of *G* in the reaction mixture must have been about three times that of *E*. At the concentration employed, 0·32 %, the latter would largely control the rate of maltose formation, as may be deduced from the affinity constants. We should therefore expect *G* to be hydrolyzed at something like three times the

rate of *E*, but it was hydrolyzed at about half the rate. With decreasing chain length the rate of β-amylase action falls off more than proportionately.

It appears that β-amylase combines with the substrate, and as regards the initial velocity of the reaction it conforms with the Michaelis & Menten theory. Owing, however, to the non-homogeneous nature of the substrate, the subsequent time progress relationship is not that usually associated with hydrolytic enzyme actions which obey the Michaelis & Menten relationship. It is a case of several substrates competing for the same enzyme.

It is assumed that the affinity constant, K_m, varies according to the mean chain length of the amylose, and when expressed as molarity decreases with increase of chain length. But the effect of increase of chain length on the value of K_m, expressed as percentage, would be to increase it simultaneously in proportion to molecular weight.

To distinguish between samples of amylose of different mean chain length by β-amylase action, a low concentration of substrate is desirable so that the latter controls the reaction velocity. It was for this reason that such conditions were imposed when amyloses precipitated under different conditions, or previously hydrolyzed to various stages, were under comparison. In the latter case, samples *F* and *G* (Table 4), being partly hydrolyzed, must have had relatively low mean chain lengths, and therefore relatively high molar concentrations compared with *E*. Nevertheless, they were hydrolyzed much more slowly. This experiment, moreover, eliminated the complication of inhibition of enzyme action by maltose.

We may therefore conclude that β-amylase attacks more readily the longer chain molecules and, provided no denaturation has taken place, hydrolysis proceeds at a steadily declining rate with no sudden change, continuing until 100% maltose has been formed.

When amylose is hydrolyzed by α-amylase or acid, the iodine coloration passes from the blue through purple and red to colourless. We have results indicating that the colourless stage corresponds to dextrins composed of about 6 glucose units, and the red stage to 10 units or so. This is supported by certain observations on synthetic products reported by Cori, Swanson & Cori (1945). When, however, β-amylase is used some blue colour remains until the hydrolysis has advanced well beyond 90%.

The relationship between the blue value and mean chain length of amylose is indicated by the results in Table 5. Whilst blue value is zero for short chains, it becomes measurable at about chain length 12, and increases rapidly with chain length. Later it approaches an asymptotic value (cf. specific optical rotation). Conversely, as the chain length is reduced by β-amylolytic action, blue value declines but little during the first 70% hydrolysis, but later falls more rapidly, disappearing at about 95%. The red value is slightly later in falling, otherwise its behaviour is similar. Most, if not all, of the red value of the original amylose is due to the same colouring matter as the blue value and is numerically proportional to it. The ratio 608 : 603 reading remains approximately constant for 75% of the hydrolysis, which would scarcely be expected if there were present in the original amylose a separate fraction such as short-chain material, giving a red colour with iodine. However, at the point where material giving blue with iodine disappears through hydrolysis, the blue value would rapidly decline, whilst the corresponding decrease in red value would be delayed. Indeed, the latter might in certain circumstances increase numerically for a time, depending on whether short-chain material giving a red iodine colour were formed fast enough to more than counterbalance the loss of red colour due to destruction of iodine-blueing polysaccharide. In at least one experiment a small rise in red value at about 80% was observed. However, owing to the possibility of appreciable error creeping in when computing the concentration of residual polysaccharide at late stages of the hydrolysis, neither the blue nor the red values in these late stages should be taken as accurate. Their ratios are significant, since the blue and red readings were taken on the same diluted material, and the above source of error is cancelled out. The ratio of 608 : 603 reading falls markedly after 80% hydrolysis, indicating the disappearance of amylose of more than 12 units chain length and generally supporting the conclusions drawn above. Apparently 80–90% or more of amylose is hydrolyzed before the mean chain length is as low as 10–12. Some specimens of amylose gave somewhat different curves from those drawn from the data in Table 5 although of the same type. Sometimes there was no appreciable fall in blue or red value till 80% hydrolysis. Sample *K* in Table 5 was a slightly 'denatured' amylose. The residue, when just over 90% hydrolysis was attained, possessed no blue or red colouring power, and was able to resist hydrolysis.

SUMMARY

1. Pure potato amylose is hydrolyzed by β-amylase at a steadily declining rate, without sudden velocity changes, until 100% of maltose has been formed.

2. If any 'denaturation' of the amylose has occurred, due to undue exposure to water, there is a break in the progress curve and the hydrolysis is incomplete.

3. Amylose crystallized from potato starch paste at pH 4, or from autoclaved paste at ordinary reaction, hydrolyzes faster than preparations crystallized from non-autoclaved pastes at pH 6 or 9.

4. The reaction obeys the formulation of Michaelis & Menten, but the value of K_m (expressed as percentage) varies widely with the preparations.

5. The shorter the molecular chains the more slowly they are hydrolyzed.

6. Evidence from iodine colorations suggests that amylose prepared by the crystallization methods contains no short-chain molecules (chain length < 12), and that none is formed until hydrolysis is far advanced.

7. The blue value of amylose increases with chain length, approaching an asymptotic value. Conversely, it decreases on hydrolysis.

8. A hypothesis is suggested to account for the observed kinetics.

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The Distribution and Interrelationships of the Alkaloids in the Bark of *Cinchona Ledgeriana*

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The literature on the interrelationships between the principal alkaloids and their distribution throughout the bark of trees of the cinchona species is scattered and rather sparse. The references available indicate that in general the alkaloid content is greater in the root bark and at the base of the tree than in the upper parts. Hodge (1946) found, however, that the reverse condition obtains in some wild Peruvian cinchonas. Sando (1944), who studied alkaloidal distribution in *Cinchona succirubra*, found a positive correlation between alkaloid content and tree girth.

The four main crystallizable alkaloids in the bark are the two groups of stereo-isomerides quinine and quinidine, and cinchonidine and cinchonine. Quinine and quinidine contain a methoxyl group in the 6-position of the quinoline nucleus, otherwise the molecular structures are the same. Such similarity in structure suggests a common mechanism of formation. The proportions in which the alkaloids are laid down in the bark, the wide differences in the proportions of the alkaloids according to bark species, and the smaller differences found among barks of the same species, and in the bark from various parts of a single tree are probably due to the operation of factors affecting the rates of conversion of one alkaloid to another.

The present investigations were undertaken to determine the distribution of the alkaloids (total, individual and amorphous) throughout the whole bark, and to relate this distribution with the form of the tree, the metric characteristics of the bark and the proportions of the various alkaloids. Some interesting relationships have been established.

EXPERIMENTAL

Materials

The trees selected were of the Ledger species, approximately 7 years old and grown on the same plantation. Bark from *C. Ledgeriana* is the richest in quinine of all cinchona barks, and contains usually 8–12% of alkaloids, of which 3–8% is quinine.

Tree 2137 was felled at ground level and the trunk sawn into 25 cm. sections over a length of 4 m. from the base. The main stem was divided into two, 2 m. from the base. The barks from sections equidistant from the base along each of these stems were combined. The bark from each section was air-dried, weighed and ground to pass a 50-mesh sieve. The root bark was collected as one sample. The sections were numbered consecutively from the base upwards.

Tree 1970. The preliminary investigations on tree 2137 showed that the alkaloid distribution is to some extent a function of the form of the tree. A detailed drawing was therefore made of tree 1970 showing the location of secondary leaders (erect growing stems as opposed to more or less horizontal branches) as well as of living, dead and, as far as possible, former branches. The tree was of irregular girth with maximum trunk thickness about 130 cm. above ground, where there was also a marked bending of the trunk from the vertical for a distance of about 20 cm. (Fig. 1).

The main stem and the secondary leaders were cut into 10 cm. sections. The lateral branches were divided into four groups (primaries, secondaries, tertiaries and quaternaries) and the leaves into seven groups ranging from very young to mature. In the lower parts of the tree one section was selected at random from each 50 cm. length, except where the formation of the tree (as revealed by the preliminary

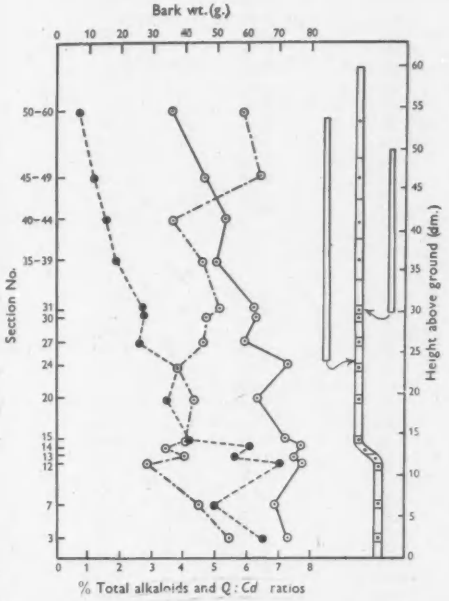

Fig. 1. Tree 1970. Diagram showing the form of the tree with the locations of the secondary leaders and the sections analyzed. The corresponding analytical data/10 cm. section are plotted on the left. ● - - - - - ● Bark weight; ⊙——⊙ percentage total alkaloids; ⊙-·-·-⊙ *Q* : *Cd* ratio. Average values of bark weight/10 cm. section are given for each of the four groups of composite samples above section 35.

work on tree 2137) was of special interest, e.g. where the main stem bent, or where big lateral branches arose. At such positions two or three contiguous sections were taken. Where the amount of bark from a 10 cm. section was insufficient for analysis, the bark from a sequence of five sections was bulked, i.e. a 50 cm. length of stem. For the lateral branch bark and leaves a composite sample was taken from each of the groups into which they had been divided. Prior to preparing the samples for analysis, the thickness, area, volume and weight (air-dry) of the bark from each section were measured; also the girth of each section and the corresponding areas of the trunk.

Tree 1512 was selected, in contrast to tree 1970, for its straight form and apparently normal growth (Fig. 2). It had produced one secondary leader only. A detailed drawing was made of the tree and the same sampling technique employed as that for tree 1970.

Analytical methods

The samples were analyzed for total alkaloids (*TA*), quinine (*Q*), cinchonidine (*Cd*), cinchonine (*C*), and amorphous alkaloids (*Am*), and the results expressed as a percentage of the moisture-free bark. Quinidine was present in traces only and was not quantitatively determined.

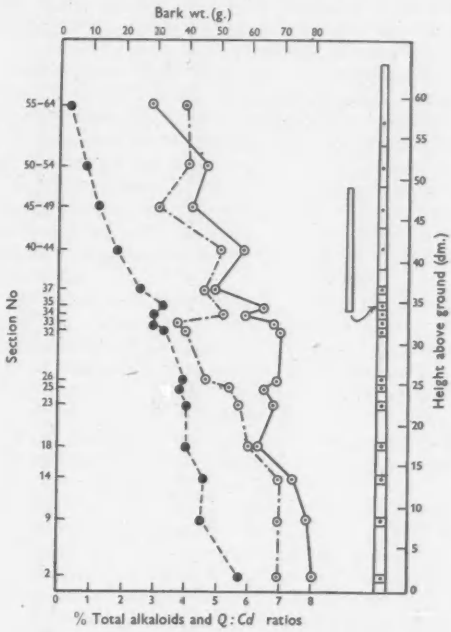

Fig. 2. Tree 1512. Diagram showing the form of the tree with the locations of the secondary leader and the sections analyzed. The corresponding analytical data/10 cm. section are on the left. ● - - - - ● Bark weight; ⊙——⊙ percentage total alkaloids; ⊙-·-·-·-⊙ *Q* : *Cd* ratio.

Total alkaloids. Air-dry bark (20 g.) was mixed with CaO (12 g.), water (15 ml.) and 5 N-NaOH (2 ml.). After standing overnight the mixture was extracted with benzene in a Soxhlet apparatus. The alkaloids were recovered from the benzene extract with 5% (w/v) H_2SO_4; the latter was then made alkaline and the liberated alkaloids extracted with chloroform. The bulk of the chloroform was distilled, and the residual extract washed with chloroform into a small tared beaker. After the addition of a small quantity of ethanol the solution was evaporated and dried to constant weight at 100°.

The individual alkaloids in the total alkaloids were determined by the *British Pharmacopoeia* (1932) method for totaquina.

RESULTS

The analytical data not shown in the figures are given in Table 1.

Table 1. *Analytical data for trees* 2137, 1970, 1512

Tree no.	Bark section no.	Alkaloids (% dry bark) Quinine	Cinchonidine	Cinchonine	Amorphous alkaloids
2137	1	6·75	1·20	0·70	1·11
	2	7·44	1·19	0·65	1·09
	3	6·99	1·11	0·70	1·00
	4	7·48	1·41	0·96	1·08
	5	7·32	1·30	0·80	0·82
	6	6·67	1·06	0·74	1·38
	7	6·82	1·22	0·90	1·17
	8	5·87	1·93	1·47	1·25
	9	6·51	1·47	0·90	0·86
	10	6·21	1·35	0·93	0·96
	11	5·31	1·38	0·78	0·90
	12	5·99	0·85	0·62	1·01
	13	6·21	1·42	0·89	1·03
	14	6·01	1·19	0·83	0·85
	15	5·06	0·62	0·71	1·04
	16	5·14	0·97	0·67	0·99
	Root bark	5·68	1·60	1·81	1·22
1970	3	4·86	0·80	Trace	1·16
	7	4·58	0·92	Trace	0·97
	12	4·64	1·47	0·22	0·97
	13	4·77	1·09	0·31	0·88
	14	4·84	1·26	0·29	0·86
	15	4·23	0·94	0·11	1·30
	20	3·18	0·67	0·09	1·78
	24	4·66	1·11	0·19	0·82
	27	2·91	0·58	0·09	1·75
	30	3·07	0·59	0·11	1·92
	31	2·94	0·53	0·13	1·86
	35–39	2·86	0·56	0·10	1·00
	40–44	2·41	0·59	0·08	1·87
	45–49	1·82	0·26	0·02	1·39
	50–60	1·79	0·28	0·03	0·96
1512	2	5·38	0·71	Trace	0·91
	9	4·79	0·63	Trace	1·26
	14	3·77	0·50	Trace	1·19
	18	3·07	0·46	Trace	1·43
	23	2·61	0·42	Trace	2·04
	25	1·62	0·27	Trace	2·31
	26	3·50	0·69	Trace	1·90
	32	3·07	0·70	Trace	1·54
	33	2·63	0·63	Trace	2·08
	34	2·56	0·45	Trace	1·38
	35	—	—	—	—
	37	2·69	0·53	Trace	1·04
	40–44	2·40	0·43	Trace	1·43
	45–49	1·34	0·39	Trace	1·30
	50–54	1·21	0·27	Trace	1·80
	55–64	0·64	0·15	Trace	0·97

Tree 2137. The distribution of the total alkaloids in the bark is shown in Fig. 3. There was an overall but irregular decrease of total alkaloid content from the base of the tree upwards. The maximum alkaloid content was found 2 m. from the base (section 8) where the tree forked, with secondary maxima (sections of high alkaloid content relative to adjacent sections) at sections 4 and 13. Unfortunately, nothing had been recorded of the form of this tree at these levels.

Fig. 3 shows also a positive correlation between percentage total alkaloids and bark weight/section. The regression equations (Table 2) show that the percentage of total alkaloids, as well as the percentages of quinine and cinchonidine, were positively and significantly related to bark weight. The regression of cinchonine on bark weight was possibly significant, and that of amorphous alkaloids not significant.

These equations are more fully discussed later, but it should be noted here that increases in bark weight were associated with significant increases in the percentages of each of the crystallizable alkaloids. The equations give, however, only a general picture of

the association between increasing bark weight and increasing alkaloid content. When the molecular ratios quinine to cinchonidine ($Q:Cd$) and quinine to cinchonine ($Q:C$) were calculated for each section, an inverse relationship was found between these ratios and bark weight/section (Fig. 3).

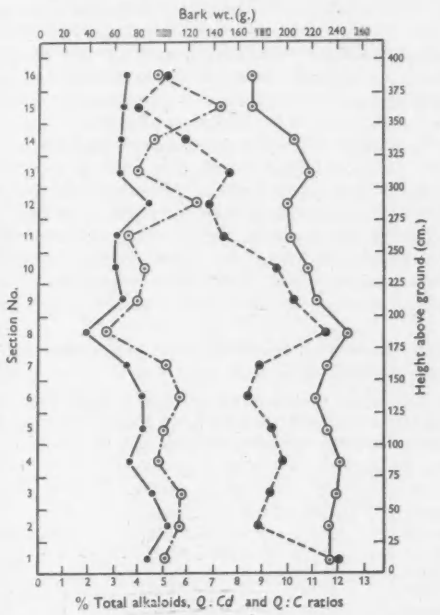

Fig. 3. Tree 2137. The relationships between bark weight, percentage total alkaloids, the quinine to cinchonidine ($Q:Cd$) and quinine to cinchonine ($Q:C$) ratio/25 cm. section of bark. The $Q:C$ ratios shown are half the true values. ●- - - -● Bark weight; ⊙———⊙ percentage total alkaloids; ⊙–·–·–·–⊙ $Q:Cd$ ratio; ●———● $Q:C$ ratio.

There was a marked similarity in the alkaloid contents of the root bark, and the bark where the tree forked. In these samples the percentages of cinchonidine and cinchonine were much higher, and the $Q:Cd$, and $Q:C$ ratios much lower than in samples from any other part of the tree. The quinine contents at the two locations were also similar. Quinine content was at a maximum in the bark from the lower stem roughly at about 1 m. above the base. The amorphous alkaloid content showed little variation throughout the length of the tree.

Tree 1970. The form of this tree is shown in Fig. 1 with the distribution of the total alkaloids, the weight of bark from the various sections and the $Q:Cd$ ratios. As with tree 2137 the decrease of alkaloid content from the base of the tree up was irregular, and wide fluctuations were associated with certain parts of the tree, in this case notably where the trunk bent and where the lower secondary leader arose. Zones of low alkaloid content were found at those parts of the trunk where there was no indication that branches were ever formed.

The regression equations (Table 2) show that the percentage of total alkaloids was positively and significantly related to the weight, thickness and volume of the bark. The percentages of quinine and cinchonidine, and possibly that of cinchonine, were likewise related to bark weight and thickness, while a negative regression, possibly significant, was found for the amorphous alkaloids. When the regressions of the percentage individual alkaloids/section on percentage total alkaloids/section were calculated, significant positive regressions were found for the three crystallizable alkaloids, and a not significant negative regression for the amorphous alkaloids (Table 2).

A further analysis of the data showed significant negative regressions for amorphous alkaloids on quinine and on cinchonidine, and very significant positive regressions for cinchonidine on quinine and cinchonine on cinchonidine (Table 3). When the individual alkaloids were expressed as percentage of the total alkaloids, the two negative regressions of amorphous alkaloids on quinine and on cinchonidine were very significant (Table 3).

With this tree, as with tree 2137, the $Q:Cd$ and $Q:C$ ratios were inversely related to bark weight or thickness (Fig. 1). A well-marked inverse relationship between bark weight and the $Cd:C$ ratios was also noted, an observation not made with tree 2137, throughout the length of which the $Cd:C$ ratios in the bark were fairly constant. The highest percentages of cinchonidine and cinchonine, and the lowest $Q:Cd$ and $Q:C$ ratios, were found where the tree bent and where the lower secondary leader arose. At the same places the quinine to amorphous alkaloid ratios were highest. The greatest percentages of quinine were also found here and at the base of the tree.

In the lateral branches there was a rapid fall in the alkaloid content of the bark, from the primaries to the tertiaries from 2·19 to 0·46% for total alkaloids, from 0·68 to 0·11% for quinine, and from 0·29 to 0·09% for amorphous alkaloids. Large-scale extractions of the leaves, separated into groups ranging from young to mature, showed total alkaloid contents varying between 0·055 and 0·223%. No correlation was found between age of the leaf and total alkaloid content, and no crystallizable alkaloids could be isolated.

Tree 1512. The shape of this tree, the distribution of the total alkaloids, the related bark weights/section, and the corresponding $Q:Cd$ ratios are shown in Fig. 2.

Taken in conjunction, Figs. 1 and 2 show how total alkaloid distribution is related to the form of the tree. In the lower half of tree 1512 the decrease in alkaloid content from the base upwards was, with relatively minor fluctuations, a gradual one. The first evidence of any living branches was at section 32, and between this and section 34, where there were no branches, the alkaloid content fell rather rapidly, but rose again at section 35 where the only secondary leader arose. Thereafter the alkaloid content decreased, but with two secondary maxima for the bulk samples taken from sections 40–44 and 50–54. These two sections had, on an average, a larger number of lateral branches than sections 45–49.

The regressions of percentage total alkaloids/section on bark weight, thickness and volume/section (Table 2) were of the same high order of significance as those for tree 1970. The interrelationships between bark weight, tree form and total alkaloid distribution appear therefore to be fairly well established.

Increases in bark weight were also associated with significant increases in the percentages of quinine and cinchonidine, but the increase in amorphous alkaloids was not significant (Table 2). Increases in percentage quinine were accompanied by significant increases in cinchonine, but no significant relationship was found between these two alkaloids and the amorphous alkaloids (Table 3). When, however, the alkaloids were expressed as a percentage of the total alkaloids a highly significant negative regression was found for amorphous alkaloids on quinine, and a possibly significant negative regression for amorphous alkaloids on cinchonidine (Table 3).

In contrast to trees 1970 and 2137 the bark from tree 1512 contained insufficient cinchonine for a quantitative determination. Moreover, the $Q : Cd$ ratios of the samples taken from the lower half of the tree decreased from the base of the tree upwards. At sections 26, 32 and 33, where the $Q : Cd$ ratios were relatively low, there were, respectively, two dead branches, two living branches and a dead and

Table 2. *Regressions of the total and individual alkaloids on bark weight, thickness and volume, and of the individual alkaloids on total alkaloids*

(Y_{TA}, Y_Q, etc. are the calculated percentages of total alkaloids, quinine, etc. per section for any value of X (bark wt., g.), X_1 (bark thickness, mm.), X_2 (bark volume, ml.) or X_3 (percentage total alkaloids), per section, lying within the range of values found for the sections analyzed. P shows the level of significance of the regression. N.S. not significant.)

	Tree no. 2137 (25 cm. section)	P	Tree no. 1970 (10 cm. section)	P	Tree no. 1512 (10 cm. section)	P
Y_{TA}	$7{\cdot}14+0{\cdot}0223\ X$	0·01	$4{\cdot}220+0{\cdot}0565\ X$	0·01	$3{\cdot}47\ +0{\cdot}084\ X$	0·01
Y_Q	$4{\cdot}45+0{\cdot}0114\ X$	0·01	$1{\cdot}645+0{\cdot}0523\ X$	0·01	$0{\cdot}371+0{\cdot}077\ X$	0·01
Y_{Cd}	$0{\cdot}42+0{\cdot}0048\ X$	0·01	$0{\cdot}210+0{\cdot}0154\ X$	0·01	$0{\cdot}256+0{\cdot}007\ X$	0·01
Y_C	$0{\cdot}45+0{\cdot}0022\ X$	0·10	$0{\cdot}031+0{\cdot}0024\ X$	0·10	—	—
Y_{Am}	$0{\cdot}90+0{\cdot}0008\ X$	N.S.	$1{\cdot}654-0{\cdot}0096\ X$	0·10	$1{\cdot}453+0{\cdot}002\ X$	N.S.
Y_{TA}	—	—	$0{\cdot}88\ +1{\cdot}0885\ X_1$	0·01	$0{\cdot}40\ +1{\cdot}320\ X_1$	0·01
Y_{TA}	—	—	$4{\cdot}25\ +0{\cdot}0162\ X_2$	0·01	$3{\cdot}16\ +0{\cdot}036\ X_2$	0·01
Y_Q	—	—	$-1{\cdot}870+0{\cdot}864\ X_3$	0·01	—	—
Y_{Cd}	—	—	$-0{\cdot}814+0{\cdot}253\ X_3$	0·01	—	—
Y_C	—	—	$-0{\cdot}170+0{\cdot}046\ X_3$	0·05	—	—
Y_{Am}	—	—	$1{\cdot}914-0{\cdot}098\ X_3$	N.S.	—	—

Table 3. *Regressions of the individual alkaloids per section. A for the alkaloids expressed as percentage of the bark, B for the alkaloids expressed as percentage of the total alkaloids*

(Y_{Am}, Y_{Cd}, etc. are the calculated values for percentage of amorphous alkaloids, cinchonidine, etc. per bark for any value of quinine (X_Q), cinchonidine (X_{Cd}) or cinchonine (X_C) percentage bark lying within the range of values found for the sections analyzed. Similarly with Y_{Am}', X_Q', etc., but these are expressed as a percentage of the total alkaloids. P shows the level of significance of the regression. N.S. not significant.)

A	Tree no. 1970	P	Tree no. 1512	P
Y_{Am}	$2{\cdot}006-0{\cdot}198\ X_Q$	0·05	$1{\cdot}754-0{\cdot}091\ X_Q$	N.S.
Y_{Am}	$1{\cdot}796-0{\cdot}640\ X_{Cd}$	0·05	$1{\cdot}602-0{\cdot}206\ X_{Cd}$	N.S.
Y_{Am}	$1{\cdot}466-1{\cdot}419\ X_C$	0·30	—	—
Y_{Cd}	$-0{\cdot}216+0{\cdot}278\ X_Q$	0·01	$0{\cdot}183+0{\cdot}109\ X_Q$	0·01
Y_C	$-0{\cdot}040+0{\cdot}044\ X_Q$	0·10	—	—
Y_C	$-0{\cdot}034+0{\cdot}196\ X_{Cd}$	0·01	—	—
B				
Y_{Am}'	$71{\cdot}11\ -0{\cdot}881\ X_Q'$	0·01	$48{\cdot}57\ -0{\cdot}533\ X_Q'$	0·01
Y_{Am}'	$44{\cdot}48\ -1{\cdot}910\ X_{Cd}'$	0·01	$39{\cdot}29\ -1{\cdot}750\ X_{Cd}'$	0·10
Y_{Am}'	$27{\cdot}15\ -2{\cdot}820\ X_C'$	0·10	—	—
Y_{Cd}'	$-4{\cdot}25\ +0{\cdot}287\ X_Q'$	0·01	$4{\cdot}023+0{\cdot}088\ X_Q'$	0·02
Y_C'	$-0{\cdot}34\ +0{\cdot}038\ X_Q'$	0·40	—	—
Y_C'	$-0{\cdot}82\ +0{\cdot}222\ X_{Cd}'$	0·02	—	—

a living branch when the tree was felled. At section 34, where the $Q : Cd$ ratio was relatively high, there were no branches. In the upper half of the tree, where many living branches joined the main stem, there was a marked fluctuation in the $Q : Cd$ ratios. The highest quinine contents were found in the three sections taken from the lowest quarter of the tree, roughly between ground level and 1·5 m.

In the lateral branches there was a rapid fall in the total alkaloid content of the bark from the primaries to the quaternaries from 2·71 to 0·68 %. Large-scale extractions of the leaves separated into groups ranging from young to mature showed total alkaloid contents ranging from 0·16 to 0·33 %, but no correlation was found between leaf age and total alkaloid content. The combined total alkaloids (1 g.) from the leaves gave a tartrate equivalent to 0·0156 % quinine plus cinchonidine in the leaves, but neither quinine nor cinchonidine could be isolated from the decomposed tartrate.

DISCUSSION

The distribution of the alkaloids in the stem bark is a function of bark thickness. In general the thickness of the bark at any point is inversely proportional to the distance of that point from the base of the tree, but a regular decrease of bark thickness from the base upwards is rare. In these investigations local thickening of the bark was found where the main stem forked, usually caused by damage of the stem at these points at an earlier stage in the tree's history, where secondary leaders arose, probably due to a similar cause, and where large branches joined the stem. Superimposed, therefore, on what is intrinsically a regular decrease in alkaloid content from the base of the tree upwards, are zones of relatively high alkaloid content, the locations of which are governed in each tree by the history of its growth as reflected in its form.

Table 2 shows, for the stem bark as a whole, the increases or decreases in alkaloid content that had been associated with unit increase in bark weight during the life of the tree. Significant increases were found for total alkaloids, quinine and cinchonidine/unit increase in bark weight, and for quinine cinchonidine and cinchonine/unit increase in total alkaloids. These increases were large compared with the small and insignificant increases (trees 2137 and 1512) and decreases (tree 1970) found for the amorphous alkaloids, an indication of the progressive conversion of amorphous to crystallizable alkaloids during the life of the tree. When the trees were felled the amorphous alkaloid content was that currently available for transformation to the crystallizable alkaloids, while the crystallizable alkaloid content represented the accumulation of the end products of the amorphous alkaloid transformations throughout tree growth.

The negative regressions shown in Table 3 lend support to the theory that the amorphous alkaloids are the precursors of the crystallizable alkaloids, additional evidence for which is furnished by observations made here that the bark of young trees, or from the younger upper parts of an old tree, contain a high proportion of amorphous in relation to crystallizable alkaloids.

The proportions in which quinine, cinchonidine and cinchonine are laid down must depend on the conditions obtaining in the bark when the amorphous alkaloids are converted. If the crystallizable alkaloids were formed simultaneously and in definite proportions, their ratios would be constant throughout the bark irrespective of its position on the tree. It was found, however, that the $Q : Cd$ and $Q : C$ ratios were notably low in the root bark and in the bark where the main stem forked, where secondary leaders arose, and where there was abnormal bark thickening. These low ratios do not imply a low quinine content, but rather a relatively greater increase of cinchonidine and cinchonine compared with that of quinine. It is evident that there is a preferential formation of cinchonidine and cinchonine where there is increased cambial activity following some form of injury or other stimulus, further evidence for which is furnished by the almost complete lack of cinchonine in the normally growing tree 1512. These observations, in conjunction with the relationships already established between bark thickness, total alkaloid content and abnormal bark growth, indicate that cambial activity plays a major role in determining the distribution of the total alkaloids in the bark, and the proportions in which the crystallizable alkaloids are laid down. Cambial activity may further govern the efficiency or degree of conversion of the amorphous to the crystallizable alkaloids, for in tree 1970 the highest crystallizable to amorphous alkaloid ratios obtained where the tree bent and where the secondary leader arose.

Botanical investigations in Java (private communication) have shown that a high quinine content is found where vertical growth is stunted and that in such areas *helopeltis* attacks are common, which further restrict growth and increase the quinine content. While a valid comparison of the average percentages of the individual alkaloids in the three trees here investigated is not strictly admissible (since the bark samples were not selected purely at random), it appears from the analytical data that tree damage was associated with an increased percentage of crystallizable alkaloids and a decreased percentage of amorphous alkaloids as compared with those in the normal tree, a further indication of some relationship between cambial activity and the transformation of amorphous to crystallizable alkaloid.

The high percentages of total alkaloids associated with local bark thickening suggests an increased translocation of amorphous alkaloids or their precursors to these places, possibly as soluble salts. The low $Q : Cd$ and $Q : C$ ratios coincident with abnormal thickening, and the variation in the proportions of the individual alkaloids and their ratios from bark section to bark section, indicate that the crystallizable alkaloids, once formed, are not further translocated. Their movement seems to be restricted towards the outer periphery of the bark as it ages and thickens, while further transformations of amorphous to crystallizable alkaloid take place in the newer underlying bark. It is unlikely that the proportions in which the alkaloids are laid down in any particular section of bark remain constant during tree growth. The association between decreasing $Q : Cd$ ratio and increasing height of bark above ground level found in the lower half of the normal tree suggests that, at least under normal conditions of growth, the transformation of amorphous to crystallizable alkaloids shifts progressively in favour of quinine as the bark ages, possibly as a result of a progressive change in cambial activity.

Dawson (1945), in his work on the physiological chemistry of the tobacco alkaloids, has demonstrated that nornicotine is produced *in vivo* from nicotine. From a consideration of their respective molecular structures he suggested that nicotine is acted upon in the cell by a transmethylating agent and a methyl acceptor (RH) and proposed as a working basis the hypothetical mechanism

$$\text{Nicotine} + R\text{H} \xrightarrow{\text{(enzyme)}} \text{Nornicotine} + R.\text{CH}_3.$$

This faculty, he considered, is inheritable.

The simultaneous increase of quinine, cinchonidine and cinchonine in the bark does not preclude the possibility that quinine may at the same time be degraded to cinchonidine and cinchonine, but at a slower rate than its synthesis from the amorphous alkaloids. The transformation may be postulated as

$$\text{Quinine} + R\text{H} \xrightarrow{\text{(enzyme)}} \text{Cinchonidine (or Cinchonine)} + R\text{OCH}_3.$$

This reaction assumes the presence of a transmethoxylating agent and a methoxyl acceptor, the reaction rate depending on the RH content of the bark, which is primarily governed by the age of the bark and its location on the tree. Such a mechanism could account for the wide fluctuations observed in the $Q : Cd$ and $Q : C$ ratios in the bark of any particular tree and, if inheritable, for the predominance of quinine in *C. Ledgeriana* and cinchonidine in *C. succirubra*.

SUMMARY

1. Alkaloid distribution in the bark of three trees of *Cinchona Ledgeriana* has been investigated. Two of the trees had suffered damage at an earlier stage of their growth and differed markedly in shape from the third tree which was of apparently normal growth and straight in form.

2. Significant positive regressions were found for percentage total alkaloids on bark weight, thickness and volume. Increases in bark weight and total alkaloids were associated with significant increases in quinine, cinchonidine and cinchonine at the expense of the amorphous alkaloids.

3. Local thickening of the bark was found where the tree forked, where secondary leaders arose and where the tree had been damaged. At these places the alkaloid content was notably high and the quinine : cinchonidine ($Q : Cd$) and quinine : cinchonine ($Q : C$) ratios relatively low. Similar conditions obtained in the root bark. Local thickening was less pronounced with the normal tree, the bark of which showed, from the ground upwards, a fairly regular decrease in the percentage of total alkaloids and in the $Q : Cd$ ratios, in contrast to the wide fluctuations observed with the other trees.

4. Meristematic or cambial activity appears to be an important factor in determining the alkaloid content of the bark, the degree of conversion of the amorphous to the crystallizable alkaloids, and the proportions in which the latter are laid down. The distribution of the alkaloids throughout the bark is therefore primarily governed by the history of the tree as reflected in its form.

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The Effect of some Thiol Compounds upon Trypsin, Chymotrypsin and Chymotrypsinogen

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With an Addendum on

Examination of Chymotrypsin in the Ultracentrifuge

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(*Received* 21 *October* 1947)

In the course of a research with another objective during the war in 1943–4, we found that thiol-containing compounds inactivated trypsin and chymotrypsin. This was not reported then. In the interval, Grob (1945–6) has published work upon trypsin done independently in 1943 and with somewhat similar observations. The work which we have now completed was carried out upon crystalline chymotrypsin and trypsin and with a different criterion for digestion; some observations have been added upon the behaviour of chymotrypsinogen. Our results for trypsin are in substantial agreement with those of Grob, though our interpretation of the phenomenon is different.

EXPERIMENTAL

Trypsin and chymotrypsin were prepared from ox pancreas by the methods of Kunitz & Northrop (1935–6) and stored in a desiccator at 2°. The trypsin used was crystalline, but had not been recrystallized; it contained 32·6% enzyme protein, accepting the usual figure for the N content of trypsin as 16%. The chymotrypsin had been once recrystallized and had a protein content of 73·7% (N, 15·5%). Ultracentrifugal examination by Mr R. Cecil (see Addendum) showed that the specimen was homogeneous. The chymotrypsinogen investigated had been reprecipitated (protein content 60·2%). It was part of the specimen used in preparation of chymotrypsin; no attempt was made to separate different forms of chymotrypsin (Kunitz, 1938–9). The activity of both enzymes was estimated by determining the extent of digestion of casein using micro-Kjeldahl estimations of N not precipitable by trichloroacetic acid (N.P.N.).

For preparation of the casein substrate, the method essentially followed that of Northrop & Kunitz (1932–3); Casein (Glaxo A/E, 50 g.) was stirred into 0·1 M-phosphate buffer, pH 7·6 (1 l.), approx. 5 N-NaOH (3–3·5 ml.) was added and the mixture heated on the water bath for 30 min. with additions of 5 N-NaOH to maintain the pH at 7·6. After cooling and removal of undissolved casein by centrifuging, the solution was kept in the refrigerator, thymol being added. Values for the preformed N.P.N. varied from 1 to 2 mg./6 ml. casein substrate; different batches of casein substrate showed variations in digestibility.

Experiments were in all cases planned to show differences due to the action of added thiol compounds. Each was set up with a control, weighed sample of the same enzyme. As no attempt was made to homogenize the enzyme preparation, this procedure controlled possible variations in the salt content of samples taken. Usually the enzyme (1–2 mg.) was incubated in 0·1 M-phosphate buffer, pH 7·6 (1·0 ml.), for 45 min., after which 6 ml. casein substrate were added. After digestion for 40–50 min. at 38°, 1 ml. 25% (w/v) trichloroacetic acid was added; after standing for 30 min. at room temperature the precipitate was filtered and washed twice with 5 ml. 2·5% (w/v) trichloroacetic acid. After adjusting the volume to 25 ml., 5 ml. were taken for ashing by micro-Kjeldahl. Most incubations were done in narrow test tubes in air; results in N_2 were not appreciably different. Glass-distilled water was used to reduce contamination by metals to a minimum.

In the earlier experiments the thiol compound was added directly to the digestion mixture; all later experiments were done by a previous incubation of thiol with the enzyme before addition to the casein. The results were qualitatively similar, but the latter procedure gave a much more marked effect. Several experiments were made with crude trypsin (liquor pancreaticus, Benger) which are not included, as they are difficult to interpret (see below).

The results are expressed as the difference in N.P.N. determined by the micro-Kjeldahl method in the digests with untreated enzyme and with enzyme incubated with the substance under test, the usual unit being mg. N.P.N./6 ml. casein substrate. In the experiments of 1943–4, control values after digestion with 1 mg. chymotrypsin were usually 14–15 mg. N.P.N., and with 2 mg. trypsin approx. 8 mg. N.P.N. For trypsin a control was done with the specific substrate N^1-benzoylarginine amide.

Control experiments with suitable samples to which no thiol was added showed that during the periods of incubation less than 10% of trypsin or 5% of chymotrypsin was inactivated. There was no evidence that SH compounds had an appreciable effect on the course of digestion; the thiol concentration during digestion was one seventh of that in the incubation mixture, i.e. incubation with 0·1 M-thiol was followed by digestion at 0·014 M; the rate of inactivation is lower for the enzyme in absence of substrate at this reduced concentration of thiol (see Fig. 1), and with casein present the rate is still lower, and not more than 1–2% extra

inhibition was found in a 30 min. digestion period. If the concentration of thiol was maintained at 0·1 M throughout the incubation and digestion periods by addition of extra MTEG with the casein, it was found that extra inhibition occurred, not exceeding 10%, interpreted as due to further inactivation by thiol of the enzyme itself. In one experiment upon trypsin, in which the thiol was removed by oxidation prior to digestion, the results were in agreement with this view. Hence we have no evidence that thiol compounds were inhibiting the course of digestion in these experiments, and it seems logical, therefore, to describe our effects as due to inactivation.

RESULTS

Inactivation of the proteinase activity of trypsin or chymotrypsin, or of both, has been shown by the following SH compounds: monothioethyleneglycol (MTEG), cysteine ester hydrochloride, glutathione,

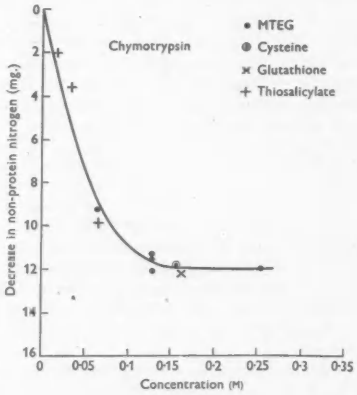

Fig. 1. Effect of previous exposure to varying concentrations of thiol compounds on the digestion of casein substrate by crystalline chymotrypsin. Addition of 1 mg. enzyme preparation to 6 ml. casein solution, pH 7·6, 38°. Digestion time 45 min. Results expressed as decrease in mg. of N.P.N. formed.

2:3-dimercaptopropanol (BAL), sodium sulphide and thiosalicylic acid; slight inactivation (14%) was produced by sodium cyanide (0·2 M) at pH 7·8; no inactivation was produced by oxidized monothioethyleneglycol (0·25 M), cystine ester hydrochloride (0·07 M), thiosulphate (0·2 M on chymotrypsin), sodium diethyldithiocarbamate (0·09 M) or pyrophosphate (see below); also, in a different category, no inactivation was produced by lewisite oxide (0·65 mM), allantoin (0·038 M), cetyltrimethylammonium bromide (CTAB) (1 mg./ml.) or heparin (1 mg./ml.). MTEG and cysteine also had a special action upon chymotrypsinogen.

Figs. 1 and 2 show in detail the effect of increasing molar concentrations of some thiol compounds acting for 45 min. at 38° upon chymotrypsin and trypsin. At approx. 0·1 M under these conditions, there was an almost maximum inactivation. The degree of inactivation induced was approximately the same for each thiol compound; this suggests that the thiol group is the active agent.

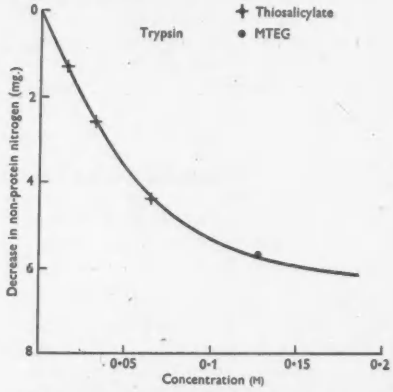

Fig. 2. Effect of previous exposure to thiol compounds on the digestion of casein substrate by crystalline trypsin. Addition of 2 mg. enzyme preparation to 6 ml. casein solution, pH 7·6, 38°. Digestion time 45 min. Results expressed as a decrease in mg. of N.P.N. formed.

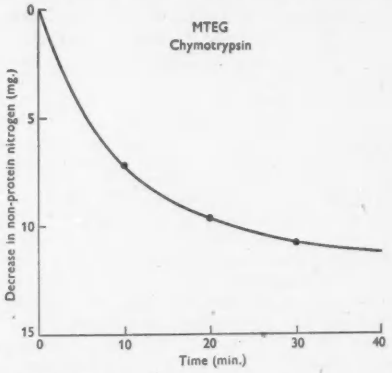

Fig. 3. Effect of time upon the inactivation of chymotrypsin activity by 0·127 M-monothioethylene glycol. Casein digest, pH 7·6. Results expressed as decrease in mg. of N.P.N. formed.

With sodium sulphide on chymotrypsin the results varied much with the alkalinity; working in tightly stoppered vessels to avoid escape of hydrogen sulphide and at pH 7·6, decreases in N.P.N. with 1 mg. chymotrypsin were observed as follows: for 0·082 M-sulphide, 4·05 mg. and for 0·125 M, 5·5 mg. N.P.N.

The relation between inactivation of 1 mg. chymotrypsin due to 0·127 M-MTEG and the time of action is shown in Fig. 3, and with this concentration the action was rapid.

The effect of varying the pH with chymotrypsin and MTEG is shown in Fig. 4, where it is clear that the action was much increased as the pH rises above 7·0; this would be expected in the case of a thiol effect.

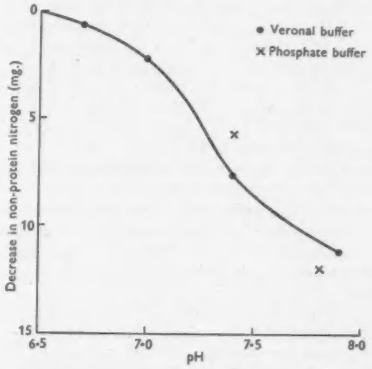

Fig. 4. Effect of varying pH on the inactivation of chymotrypsin activity by 0·127 M-monothioethyleneglycol. Digestion time 45 min. for 1 mg. enzyme added to 5 ml. casein solution, pH 7·6. Results expressed as decrease in mg. N.P.N. formed.

The inactivating effect of thiols upon the action of trypsin upon the specific substrate N^1-benzoylarginine amide (Bergmann, Fruton & Pollok, 1939) was also proved in two experiments, of which one is quoted in Table 1.

Table 1. *Inactivating effect of monothioethyleneglycol (MTEG) and 2:3-dimercaptopropanol (BAL) upon the digestion of N^1-benzoylarginine amide*

(Volumes of 0·01 N-ethanolic HCl required by 0·5 ml. substrate titrated in 90% acetone (Linderstrøm-Lang technique) before and after digestion at 38° for 2 hr. and containing initially 0·051 M-benzoylarginine amide and 0·4 mg. trypsin.)

Addition	Start (ml.)	2 hr. (ml.)	Diff. (ml.)
None	+0·08	+1·27	+1·19
MTEG (0·128 M)	+0·06	+0·05	−0·01
BAL (0·031 M)	+0·43	+0·51	+0·08

These phenomena were so much like those already well known for insulin (cf. Abel & Geiling, 1925; du Vigneaud, 1927), where reduction of a S-S group leads to inactivation, that they led to attempts to get evidence (i) that S-S compounds had no effect on these enzymes; (ii) that there was no reactivation upon reoxidation with S-S compounds; and (iii) that a SH group was present after treatment with the thiol. The results were as follows:

(i) Neither cystine ester hydrochloride (30 mg./ml.) nor oxidized MTEG (20 mg./ml.) produced any effect (Table 2).

Table 2. *Effect of oxidized MTEG, and of cystine ester* HCl *upon the activity of chymotrypsin*

(All results expressed for 1 mg. enzyme in 1 ml. added to 6 ml. casein solution. Inhibitors added at start of digestion; 38° for 45 min.)

Addition	N.P.N. formed (mg.)	Decrease in N.P.N. formed (mg.)
None	14·0	—
0·256 M-MTEG	—	4·5
0·256 M-MTEG (oxidized)	—	0·15
0·127 M-Cysteine HCl	—	2·8*
0·162 M-Cystine ester HCl	—	0·4*

* In arriving at these figures, a blank has been subtracted for the N present in the cystine or cysteine.

(ii) An attempt to reactivate chymotrypsin and trypsin, after exposure to sodium sulphide and MTEG and subsequent removal of unchanged thiol, is shown in Table 3 (*a*) and (*b*); relevant details of the experiments are given below. No reactivation was found either with cystine (as ester) or with oxidized glutathione.

The treatment of chymotrypsin in (*a*) was as follows: After exposure for 45 min. to the sulphide at pH 7·6 in a stoppered tube to prevent escape of H_2S the unchanged H_2S was removed *in vacuo* at pH 6·0 (HCl) until none was detectable by smell. Cystine ester (0·29 M, reckoned on S content) was added, the pH readjusted, and the whole incubated for a further 45 min. Casein substrate (6 ml.) was added and digestion carried on for 45 min. Trichloroacetic acid (25% w/v; 1 ml.) was then added, and the whole left to stand for 30 min. The precipitates were washed three times

Table 3. *Attempted reactivation of chymotrypsin (1 mg.) and trypsin (2 mg.) with cystine ester or oxidized glutathione, respectively, after inactivation by sodium sulphide or monothioethyleneglycol (MTEG)*

	Inactivating compound	Attempted reactivation by	Decrease in N.P.N. found (mg.)
(*a*) Chymotrypsin	0·082 M-Na_2S	—	4·05
	0·125 M-Na_2S	—	5·5
	0·082 M-Na_2S	0·29 M-cystine ester*	5·1
	0·125 M-Na_2S	0·29 M-cystine ester*	6·4
(*b*) Trypsin	0·64 M-MTEG	—	3·25
	0·64 M-MTEG	0·13 M-oxidized glutathione	3·25

* Reckoned on S content.

with 2·5% trichloroacetic acid (5 ml.) and the filtrates made up to 25 ml., 5 ml. being taken for analysis. The precipitates were left standing with 5% trichloroacetic acid (5 ml.) for 18 hr., after which the supernatant fluid gave no nitroprusside test for cystine, indicating complete extraction. The control enzyme was incubated for 1·5 hr., NaCl (12 mg./ml.) being added equivalent to that formed from Na_2S in the experimental tube.

The treatment of trypsin in (*b*) was as follows: After exposure to MTEG for 2 hr. and cooling, the solution of inactivated enzyme was saturated with $MgSO_4$ and allowed to stand in the cold. The precipitate was removed by a small filter, washed with saturated $MgSO_4$ until the washings were free from SH, and then taken up in phosphate buffer, pH 7·6. A sample was removed for estimation of activity, and the remainder treated with oxidized glutathione and incubated for 2 hr. at 38°. Further samples were then taken to estimate the activity.

(iii) Evidence of the appearance of SH groups was sought by titrating the enzymes with porphyrindin solution (Kuhn & Desnuelle, 1938) before and after treatment with monothioethyleneglycol. Mirsky & Anson (1934–5) used thioglycollic acid, but MTEG has the advantage that no COOH group is present to form possible salts. Some difficulty was experienced in making certain that any effect observed was not due to traces of MTEG absorbed on the precipitates or filter papers. After treatment with the thiol, trypsin was clearly much altered; it was no longer precipitable by 5% trichloroacetic acid, which prevented use of this reagent in washing. Two procedures were finally adopted, viz. precipitation by ammonium sulphate, and treatment with 90% acetone (Stern & White, 1937), both at acid reaction, to minimize oxidation of any thiol group. Examples of these are given below, Exp. 1 for chymotrypsin, Exp. 2 for trypsin and Exp. 3 for the parent protein chymotrypsinogen.

In these experiments, the ratio of thiol to enzyme was reduced for experimental convenience. The relation of thiol concentration to rate of inactivation has not been systematically investigated, but control experiments showed that the concentrations used in the experiments below were adequate to inactivate substantially within 30 min.

Exp. 1 (7. viii. 47). Chymotrypsin (50 mg. containing 36·8 mg. ash-free enzyme) was dissolved in 2 ml. 0·05 M-phosphate buffer, pH 7·6; 100 mg. MTEG were added and the pH again adjusted. After incubation at 38° for 120 min., the mixture was cooled, N-HCl was added to bring the pH to 3·0, and saturated $(NH_4)_2SO_4$ added to 60% of saturation. The precipitate was removed by centrifuging (2500 r.p.m.) and then transferred to a very small pressure filter (filter paper 8 mm. diam.); the precipitate was washed on the filter with saturated $(NH_4)_2SO_4$ solution until the filtrate gave no nitroprusside reaction for thiol; it was then suspended, with the paper, in 0·001 N-HCl, reprecipitated with $(NH_4)_2SO_4$ and again washed on a fresh filter paper. This operation was repeated once again. The washed precipitate of 'thiol-treated' enzyme was then suspended in phosphate buffer, pH 7·3, and immediately titrated with porphyrindin (0·5 mg./ml. standardized against cysteine ester hydrochloride) until fresh additions of 0·025 ml. did not decolorize in 30 sec. The total amount used was 1·05 ml. porphyrindin, equivalent to 0·69 mg. cysteine ester HCl. One earlier experiment gave 87% of this value.

Reckoning the S content of chymotrypsin as 1·85% (Kunitz & Northrop, 1934–5), this means that approximately one fifth (17·4%) of S had appeared as SH groups. In a control experiment run at the same time, a solution of 25 mg. of chymotrypsin, not treated with thiol during the incubation, was acidified; MTEG was then added, and the protein immediately precipitated with $(NH_4)_2SO_4$ and the washing procedure carried out as above. When dissolved in phosphate buffer this control chymotrypsin reduced less than 0·025 ml. porphyrindin solution.

In another experiment with chymotrypsin and MTEG done by the method described under Exp. 3, the chymotrypsin washed free from MTEG gave a marked nitroprusside reaction for SH upon addition of nitroprusside and ammonia.

Exp. 2 (9. viii. 47). Trypsin (50 mg.) was treated with 2·0 ml. phosphate buffer, pH 7·8; the precipitated magnesium phosphate was removed by centrifuge, and the resulting solution placed in a closed vessel (Stern & White, 1937); N_2 was passed at 38°. After 5 min. MTEG (100 mg.) was quickly added; the resultant pH was 7·6. After action for 60 min. in the N_2 atmosphere, 0·3 ml. N-HCl was added, the mixture cooled and 9 vol. of acidified acetone (0·55 ml. N-HCl to 250 ml.) added. After centrifuging, the precipitated protein was washed 6 times with the acidified acetone and dried in the centrifuge tube *in vacuo*. There was no smell of thiol. The protein was dissolved in 1 ml. phosphate buffer, pH 7·3, and immediately titrated with porphyrindin, using an amount of dye equivalent to 0·106 mg. cysteine ester hydrochloride. Reckoning 1·1% as the S content of trypsin (Kunitz & Northrop, 1934–5) this gives 10·2% of S reduced, a value which should be increased by at least one tenth to allow for losses during the initial washing.

A previous experiment with trypsin, carried out on the lines of Exp. 1 with $(NH_4)_2SO_4$ fractionation, gave 8·2% of S reduced, which is of the same order as that in Exp. 2. Trypsin (50 mg.), not treated with thiol until the washing stage, reduced less than 0·02 ml. dye.

Exp. 3 (20. viii. 47). Chymotrypsinogen (50 mg.) was treated with 2·0 ml. phosphate buffer, pH 7·8, and MTEG (100 mg.). Rapid precipitation took place, most of the protein coming out of solution in 10 min. After 30 min. at 38°, the solution was cooled on ice, the precipitated protein was removed by centrifuging, and suspended in 0·02 N-HCl (2·0 ml.); 0·2 ml. N-HCl was added and 9 vol. of cold acidified acetone. After removing the precipitate by centrifuge, it was resuspended in cold 0·02 N-HCl, 9 vol. of acid acetone added, and the precipitate washed with acetone; the resuspension and washing were repeated, and the precipitate was finally dried *in vacuo*. There was no smell of thiol. The dried product was suspended in phosphate buffer, pH 7·3 (1·0 ml.), transferred to a small test tube and titrated with porphyrindin until small additions were not decolorized in 30 sec.; the porphyrindin used was equivalent to 0·397 mg. cysteine ester HCl. Reckoning chymotrypsinogen S as 1·48% (Brand & Kassell, 1941–2), this corresponds to a reduction of 15·4% of the S present. Before thiol treatment, chymotrypsinogen showed no SH content by porphyrindin titration.

These experiments have convinced us that reducing thiol groups are formed in these proteins by the action of MTEG; they accounted for one tenth to one fifth of the total S stated to be present; but the exact amounts of thiol which can be formed must be decided by future, more accurate experiments. In this connexion Brand & Kassell (1941–2), in a very pure specimen of chymotrypsinogen, have found the content of methionine S to be 0·262 % and of cystine + cysteine S, 1·225 % ; it is not known how the S content changes upon conversion to chymotrypsin, but they record the odd point that hydrolysis with hydrochloric acid yields 4 cysteine groups/molecule; they state that no reactive SH was present in chymotrypsinogen as determined by nitroprusside and ammonia, with which our titration values agree.

Relation to denaturation. The appearance of thiol groups in these experiments is not similar to the well-known unmasking of thiol groups in the denaturation of egg albumin; in the controls mentioned, which had been treated with acid acetone, the chymotrypsin and chymotrypsinogen were denatured in the sense that the protein became insoluble, without liberation of thiol. Similarly, neither boiling the three proteins in water to which a trace of acetic acid had been added to slow down oxidation of any possibly liberated thiol, nor treatment with purified guanidine hydrochloride (6 M) (Greenstein, 1938) led to a liberation of thiol; subsequent addition of sodium nitroprusside and ammonia gave no thiol reaction, which readily appeared upon addition of minimal amounts of cyanide. The denatured proteins had a disulphide grouping and behaved exactly as denatured serum albumin (Walker, 1925).

All the evidence so far available suggests that the behaviour of trypsin and of chymotrypsin, as well as of chymotrypsinogen, to treatment with thiols is similar to that of insulin.

Attempted inactivation by pyrophosphate. In view of the statement by Grassmann, Dyckerhoff & Schoenebeck (1930) that trypsinkinase was inactivated by pyrophosphate as well as by cysteine, this has been investigated. No effect was found in two experiments; details of one are given in Table 4.

Table 4. *Effect of pyrophosphate* (pH 7·6) *upon activity of crystalline trypsin* (2 *mg.*)

(Incubated for 3 hr. with and without sodium pyrophosphate (0·224 M); casein substrate then added and digestion carried out for 40 min.)

	N.P.N. formed (mg.)
Control	3·80
Pyrophosphate	4·00

Relation to metals. As might be expected, there was no change in the degree of inactivation due to thiol produced in our experiments by addition of manganese or zinc (1 mg.) together with the thiol. The action of thiols was not an effect on metals. The activation of papain by cyanide and thiol compounds is well known, and also the pioneer experiments of Hopkins & Morgan (1938) upon the reversible inactivation of succinodehydrogenase by S-S compounds. As noted by Grob (1945–6), the only earlier reference to an inactivating action of thiol compounds upon trypsin, by Grassmann *et al.* (1930), referred to inactivation of trypsinkinase at pH 8·9 in 0·2 M-ammonia-ammonium chloride buffer solution. Using amounts of cysteine up to 0·02 M, it was found that trypsin alone was substantially inactivated; after adding intermediate amounts of kinase, the extra digestion due to kinase was inhibited, even by 0·001 M-cysteine; addition of extra kinase practically abolished the inhibition. Pyrophosphate behaved in a similar way. It is to be noted that they were using an ethanolic titration as a method of estimation. Since we found no effect of pyrophosphate, in our opinion their results would be better interpreted as being due to the action of a peptidase, present in the trypsin preparation, which was activated by some divalent metal added with the kinase. It might be expected that the effect of the metal would be removed by combination with a thiol or with pyrophosphate.

We have tried the effect of adding thiol upon the manganese activation of the peptidase which splits

Table 5. *Increasing effect of* 0·288 M-*MTEG upon the activation by manganese of aminopeptidase in a Fruton-type extract of rat skin as the manganese concentration is reduced. Substrate,* L-*leucylglycine*

(Samples of 0·5 ml., containing 4·67 mg. L-leucylglycine and 0·2 ml. skin extract, taken after digestion for titration in 90% acetone by the Linderstrøm-Lang technique. Results given are increases in 0·01 N-ethanolic HCl titrated as a result of the digestion.)

Exp.	Digestion time (38°) (hr.)	Addendum	Manganese addition: Nil (ml.)	0·0037 M (ml.)	0·00037 M (ml.)
4	3·5	Nil	0·33	2·16	—
		MTEG	0·23	1·63	—
5	2	Nil	0·38	—	1·85
		MTEG	0·01	—	1·00

L-leucylglycine in skin (Fruton, 1946; Neville-Jones & Peters, 1948), and have found a decreasing effect with the addition of larger amounts of manganese, a result much in keeping with the results of Grassmann *et al.* (Table 5).

DISCUSSION

Using smaller amounts of crystalline trypsin than in these experiments Grob found with approx. 0·01 M-cysteine, 59 % inhibition; with 0·04 M-sodium thioglycollate, 26 %; with 0·003 M-glutathione, 17 %; and with one fifth saturated H_2S solution, 7 %. These inhibitions are less complete than those noted by us. He also found a 50 % inactivation with 0·05 M-cyanide. Working at pH 7·6–7·8 we have only found slight inhibition with 0·2 M-cyanide. It is to be noted that Blatherwick, Bischoff, Maxwell, Berger & Sahyun (1927) found that, at pH 7·0, 0·4 M-cyanide took 18 hr. to inactivate insulin completely. Grob considered that his results showed a sensitivity to oxidation-reduction in the protease molecule; since we have found that there is no reactivation by exposure to S-S compounds and that the molecule seems definitely to come to pieces, we think the phenomenon is irreversible. The precipitation of chymotrypsinogen by SH action is the first case known to us in which such a change of state can be produced by reduction alone; in this it seems uniquely interesting.

It is interesting also that at least three active proteins from the pancreas are inactivated by reduction, in addition to the similar action on chymotrypsinogen. It is natural to think that inactivation by reduction may have biological importance, but for the present we have no evidence of this and must point out that the concentrations of thiol agents used here were relatively very high.

Note added 21 *October* 1947. Since the completion of this manuscript our attention has been drawn to the statement by E. C. Webb & R. van Heyningen that 0·0042 M-BAL had no effect upon a commercial specimen of trypsin (Webb & van Heyningen, 1947). The result does not conflict with our statements, as the concentration used was relatively small and the time of exposure to the enzyme alone not more than 15 min.

SUMMARY

1. Several thiol compounds, monothioethyleneglycol, cysteine, glutathione, 2:3-dimercaptopropanol, thiosalicylic acid and sodium sulphide irreversibly inactivate crystalline trypsin and chymotrypsin.

2. This action, like the well-known effect on insulin, increases on the alkaline side of the neutral point.

3. The inactivating effect is not reversible by exposure to S-S compounds, and is accompanied by the appearance of SH groups. This is not an unmasking of thiol groups by ordinary denaturation.

4. SH groups also appear in chymotrypsinogen upon treatment with some thiol compounds, and the protein is precipitated.

Our thanks are due to Mr A. Harrison for help with the preparation of the crystalline enzymes; to Dr L. A. Stocken for cystine and cysteine ester hydrochloride and for porphyrindin, and to Dr Whittaker for monothioethyleneglycol.

ADDENDUM

Examination of Chymotrypsin in the Ultracentrifuge

By R. CECIL, *Department of Biochemistry, Oxford*

A sample of chymotrypsin was examined in the Svedberg oil turbine ultracentrifuge by the method of Philpot (1938). The concentration of protein was 0·74 % (refractive increment at 546 mμ. = 0·00134) in buffer containing 0·2 M-NaCl, 0·058 M-KH_2PO_4, and 0·008 M-Na_2HPO_4. The speed was 1020 rev./sec.

The sedimentative diagram (Fig. 5) showed a single homogeneous component of S_{20} (corr.) = $3·09 \times 10^{-13}$. Integration of the area of the boundary accounted for 75 % of the total refracting material.

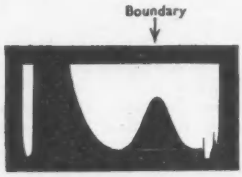

Fig. 5. Sedimentative diagram of chymotrypsin, showing the boundary 70 min. after reaching full speed.

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Quantitative Determination of Glutamine and Glutamic Acid

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Washed suspensions of *Clostridium welchii*, strain S.R. 12, as shown by Gale (1945, 1947), specifically decarboxylate L-glutamic acid and L-glutamine and are thus a specific reagent for the determination of these two substances. It is shown in this paper that the decarboxylation of glutamine is accompanied by the formation of an equivalent amount of ammonia, and that the glutaminase responsible for this reaction is highly specific. No other compound has so far been found to yield ammonia when incubated with washed suspensions of the organism at pH 4·9. This glutaminase preparation is more convenient to make than Archibald's (1944) glutaminase preparation from kidney and is more specific.

The following method for the quantitative determination of glutamic acid and glutamine rests on these facts, and combines the principles used by Gale (1945) and by Archibald (1944). The sum of the substances is determined according to Gale's procedure, slightly modified, by manometric determination of the carbon dioxide evolved on decarboxylation. The ammonia is then estimated in the solution treated with *Cl. welchii*, and in a sample not so treated. The difference represents the glutamine.

Preliminary experiments indicated that the rate of CO_2 evolution from glutamine is under some conditions considerably slower than the CO_2 evolution from glutamic acid, and a detailed study of the conditions favourable for the quantitative decarboxylation of glutamine was therefore required.

EXPERIMENTAL

Bacteria. The strain S.R. 12 of *Cl. welchii* as recommended by Gale (1945) was used for most experiments. In a few cases, a strain of the same species supplied by the Department of Bacteriology of this University and containing a histidine- and a glutamic-decarboxylase was used. The organisms were grown at 38° in the medium suggested by Gale except that 50 ml. of yeast extract were added to 1 l. medium (prepared by autoclaving baker's yeast with an equal volume of water and filtering). The washed cells from about 1 l. medium were suspended in about 15 ml. of 0·45% (w/v) NaCl. The dry weight of the bacteria in the suspension was determined in a 0·2 ml. sample. The yield from 1 l. of medium was 1·1–1·5 g. of dry bacteria. All data on bacterial weights in this paper refer to dry weight. The suspension was kept in a refrigerator where its activity did not appreciably deteriorate within a month.

Chemical determinations. CO_2 was measured manometrically with air in the gas space of the vessels, NH_3 according to the method of Parnas & Heller (see Parnas, 1934).

Chemicals. The L-glutamine used was prepared from mangel-wurzels. In spite of frequent recrystallizations from ethanol-water and water, its purity was only 93% as judged by amide-N content and CO_2 evolution. Among the impurities were about 1·3% arginine (see Archibald, 1945). The L-glutamic acid preparation was 96% pure. The impurity was probably its optical enantiomorph, as the amino-N content agreed with the theoretical value whilst Gale's method yielded 96%.

Table 1. *Rate of* CO_2 *evolution from glutamine and glutamic acid by* Clostridium welchii *at varying substrate concentrations*

(Each cup contained 2 ml. substrate solution, 1 ml. 0·2 M-acetate buffer (pH 4·9), 0·05 ml. bacterial suspension (≡ 1·1 mg.); 30°.)

Substrate ...	Glutamine				Glutamic acid			
Final substrate concentration (M) ...	0·013	0·0065	0·00325	0·001625	0·013	0·0065	0·0034	0·001625
Total amount of substrate/cup (μl.) ...	448	224	112	56	448	224	112	56
CO_2 (μl.) evolved after: 10 min.	19	12	6·5	1	34	30	17·5	13
20 min.	39	28	14	3	65	55	36	23·5
40 min.	91	58	29·5	7	122	120	65	46

Table 2. *Effect of* pH *on the rate of* CO_2 *evolution from glutamine and glutamic acid by* Clostridium welchii

(Each cup contained 3 ml. 0·1 M-acetate or lactate buffer, 1 ml. 0·01 M-substrate, 0·5 ml. bacterial suspension (11 mg.); 30°.)

Substrate ...	Glutamic acid							Glutamine						
Buffer ...	Acetate					Lactate		Acetate					Lactate	
pH ...	5·0	4·7	4·4	4·1	3·7	3·4	3·0	5·0	4·7	4·4	4·1	3·7	3·4	3·0
CO_2 (μl.) evolved after:														
5 min.	60	64	74	74	75	54	34	26·5	21·5	15	10·5	8·5	2·1	1
10 min.	154	160	168	166	143	115	74	72	57	35	22	18	5	2
15 min.	189	198	203	203	179	170	110	110	109	52	30	25·5	9	3
50 min.	211	211	216	218	208	218	203	190	184	132	82	64·5	26	12

Metabolism of glutamine in Clostridium welchii

Rate of CO_2 *evolution from glutamine.* Data comparing the rates of CO_2 evolution from glutamine, and from glutamic acid under the same conditions, are recorded in Table 1. Differences in the rates are especially marked at low concentrations of the substrates. As will be shown below, these results, obtained on pure solutions of the substrates, can be modified by the presence of other substances.

Effect of pH. Glutamic acid decarboxylase shows only small changes in activity between pH 3·4 and 5·0 (Table 2; see also Gale, 1941). The optimum is fairly broad and extends from about pH 3·7 to 4·4. At pH 3·0 (lactate buffer) the activity is still about 50% of the optimal activity. In contrast, CO_2 evolution from glutamine falls fairly rapidly with pH between 5·0 and 3·0; at pH 3·0, the rate is only 3% of that observed at pH 5·0.

Production of ammonia. Fig. 1 shows that the evolution of CO_2 from glutamine is accompanied by the production of ammonia. During the early stages of the reaction more ammonia than CO_2 is formed, but later the yields of both substances become identical. This indicates that the formation of ammonia precedes decarboxylation, as is borne out by the observation that the CO_2 evolution from glutamine, unlike that from glutamic acid, has a short lag period during which the rate of decarboxylation gradually rises (Table 2, Fig. 1). This is to be expected if the substrate of decarboxylation, glutamic acid, is not present at the start, but is gradually formed under the influence of glutaminase. Further evidence of the primary hydrolysis of the amide group is afforded by inhibitor experiments. Cyanide (3×10^{-3} M), which completely inhibits decarboxylation (Gale, 1945), but has no effect on the evolution of ammonia, separates the action of glutaminase and glutamic decarboxylase, whilst $HgCl_2$ inhibits both reactions (Table 3).

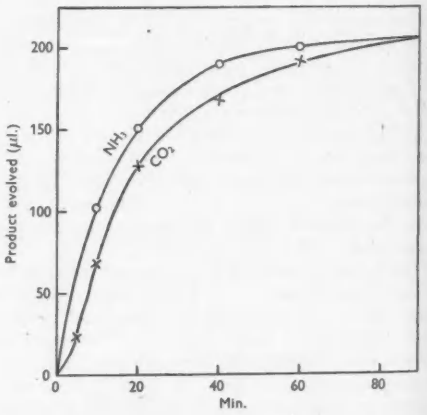

Fig. 1. Formation of NH_3 and CO_2 from glutamine in the presence of *Clostridium welchii*. 3 ml. 0·2 M-acetate buffer, pH 4·9; 1 ml. 0·01 M-glutamine; 0·5 ml. bacterial suspension (≡ 11 mg.); 30°. The reaction was interrupted by addition of 1 ml. 2 N-HCl at the specified time.

Table 3. *Effect of* HCN *and* $HgCl_2$ *on the action of* Clostridium welchii *on glutamine*

(pH 4·5, 1·46 mg. glutamine, 30°.)

	Inhibitor		
	None	3×10^{-3}M-$HgCl_2$	3×10^{-3}M-HCN
CO_2 after 60 min. (μl.)	171	2	3
NH_3 after 60 min. (μl.)	183	~Nil	190

Temperature coefficient of decarboxylation. The rate of decarboxylation of glutamine and of glutamic acid at 40° was about 60 % faster than at 30°. There was no indication that the bacterial cells suffered damage through prolonged incubation at 40°.

Effect of narcotics. Gale (1946) has already noted that ethylurethane does not inhibit bacterial decarboxylases. Other unspecific 'narcotic' inhibitors like octanol or phenylurethane (both in saturated solutions) were found not to affect the decarboxylation of glutamine.

Salts. At pH 4·9 no difference in the rates of decarboxylation was found when the concentration of the acetate buffer was varied between 0·05 and 0·2M. Higher buffer concentrations caused inhibitions, *c.* 40 % by 0·4M-buffer and *c.* 60 % by 0·8M-buffer. NaCl, in presence of 0·2M-acetate buffer, had no appreciable effect as long as its concentration did not exceed 1M (see also Taylor & Gale, 1945).

Effect of detergents. In the course of experiments on inhibitors it was observed that addition of cetyltrimethylammonium bromide ('cetavlon') accelerated the decarboxylation of glutamine. Examples are shown in Table 4. The magnitude of the effect depends, among other factors, on pH. Under the conditions shown in Table 4, the increase of the initial rate was *c.* 2·5-fold at pH 4·9, *c.* 10-fold at pH 4·1 and *c.* 20-fold at pH 3·8. Thus the shape of the pH-activity curve of the decarboxylation of glutamine is changed by the detergent, and the curve obtained with washed cell suspensions is therefore not the true pH-activity curve. Other factors, presumably the permeability of the cells, affect the rate of interaction between enzyme and substrate.

Table 5, giving the effects of varying concentrations of cetavlon, shows that 0·48 mg. detergent can appreciably increase the activity of 6 mg. bacteria and that the maximum effect is produced by about 1 mg. detergent.

The decarboxylation of glutamic acid was, under the same conditions, also acclerated by cetavlon, but owing to the relatively high rate of the decarboxylation of glutamic acid without detergent the percentage increase produced by cetavlon was smaller than in the case of glutamine. The effect of adding the detergent to the bacteria before the substrate, e.g. incubation with cetavlon for 20 min. before the addition of the substrate, was to produce inactivation (see also Baker, Harrison & Miller, 1941).

An anionic detergent, sodium dodecyl sulphate, completely inhibited the decarboxylation of glutamine under the conditions stated in Table 4, when 0·05 % detergent was added to the substrate solution.

In experiments with another strain of *Cl. welchii*, which decarboxylated histidine in addition to glutamic acid and glutamine, cetavlon likewise accelerated the glutamic decarboxylase but inhibited the histidine decarboxylase, whilst sodium dodecyl sulphate inhibited the decarboxylation of glutamic

Table 4. *Effect of cetavlon on the decarboxylation of glutamine at different* pH

(1·46 mg. glutamine in 3 ml. 0·2M-buffer; 40°; 0·1 ml. bacterial suspension (3 mg. bacteria); cetavlon added to substrate solution.)

Buffer		Acetate	Acetate	Lactate	Acetate	Acetate	Lactate
pH		4·9	4·1	3·8	4·9	4·1	3·8
Cetavlon (%)		0	0	0	0·13	0·13	0·13
CO_2 (μl.) evolved after:	5 min.	26	6	2	68	55	39
	10 min.	52	11	4	137	111	86
	15 min.	77	13	6	174	172	125
	20 min.	96	16	7	187	192	161
	30 min.	126	21	8	195	204	186

Table 5. *Effect of varying cetavlon concentrations on the decarboxylation of glutamine*

(1·46 mg. glutamine in 4 ml. 0·1M-acetate buffer, pH 4·1; 0·2 ml. bacterial suspension (6 mg.); cetavlon added to substrate solution.)

Cetavlon concentration (final) (%)		0	0·012	0·024	0·048	0·096	2·0
Total amount of cetavlon added (mg.)		0	0·48	0·96	1·92	3·84	80
CO_2 (μl.) evolved after:	5 min.	2	17	89	78	87	85
	10 min.	8	44	176	182	181	179
	15 min.	13	65	201	205	202	205

acid and glutamine and had no effect on the histidine decarboxylase. These experiments will be reported in full elsewhere.

Effect of serum. Blood serum (Table 6) also accelerated the decarboxylation of glutamine by *Cl. welchii*, the effect depending on pH. At pH 4·1, larger (*c.* 10-fold) amounts of serum were required than of cetavlon (on a w/w basis) and the maximum effects of serum were smaller. Gelatin (0·6 %) had no effect; 'Bacto' peptone (0·6 %) caused a small increase (about 30 %) of the initial rate. When both cetavlon and serum were added, the effects observed were somewhat smaller than those of cetavlon alone (Table 6). At pH 4·9, the maximum effects produced by cetavlon and by serum were about equal, and smaller quantities, about one half, were required for the maximum acceleration. Aqueous extracts of animal tissues had effects similar to those of serum.

Cetyltrimethylammonium bromide. Commercial cetavlon in water (2 %) served as a stock solution. According to the makers, cetavlon contains about 70 % of cetyltrimethylammonium bromide. A purer preparation (about 97 %), kindly supplied by Mr S. Ellingworth of Imperial Chemical (Pharmaceuticals) Ltd., did not appreciably differ from commercial cetavlon in its action on the decarboxylation of glutamine.

Manometric arrangement. Conical flasks provided with a side arm are used. The solution to be examined is placed in the main compartment. If very acid or very alkaline it is first adjusted to *c.* pH 4·9 by the addition of HCl or NaOH, and finally by 3 M-acetate buffer, usually $\frac{1}{15}$ vol. The total volume of the fluid in the main compartment should not exceed 4 ml. The side arm contains 0·5 ml. of bacterial suspension. Two types of control are required, one to measure the gas exchange of the bacteria alone, the other to measure the gas exchange of the solution to be examined. The first contains the bacterial suspension in the side arm and 4 ml. 0·2 M-acetate buffer in the main compartment. The second contains a sample of the unknown solution, treated in the same way as in the test (no bacterial suspension in the side arm). The manometers are shaken at 40°; the contents of the side arm are mixed when equilibrium is established, and readings are taken until evolution of CO_2 ceases. Glutamic acid usually reacts completely within 15 min., glutamine within 20–30 min.

The control containing bacteria alone usually gave a small negative pressure, e.g. 4 μl. in the first 30 min. and 6 μl. in the second 30 min., and it contained no more than a trace of NH_3 (4 μl.). The gas exchange in the second control, also, is often negligible. An O_2 uptake, however, may occur when tissues are examined in which the enzymes have not been inactivated (see below).

Determination of NH_3. On completion of the CO_2 evolution the cups are detached from the manometers, and NH_3 is determined in the solutions by steam distillation *in vacuo* according to Parnas (1934). A modified apparatus which allows the collection of 20 ml. of distillate was used.

Calculation. For convenience the amounts of CO_2 and NH_3 are both expressed in μl. After correction for blanks, the NH_3 produced in the unknown solution is equivalent to the glutamine, and the CO_2 to glutamine + glutamic acid.

Table 6. *Effect of serum on the decarboxylation of glutamine at* pH 4·1

(1·46 mg. glutamine in 4 ml. total volume; 0·1 M-acetate buffer, pH 4·1; 40°; 0·1 ml. bacterial suspension (3 mg.). The serum contained 7·9 % protein.)

Sheep serum added (ml.) ...	0	0·2	0·4	0·8	1·6	0	0·8
Cetavlon added (mg.) ...	—	—	—	—	—	1·6	1·6
CO_2 (μl.) evolved after: 5 min.	3	11	23	37	52	66	52
10 min.	6	20	50	86	117	149	133
15 min.	7	32	79	120	166	186	176

Procedure for the determination of glutamine and glutamic acid

Data presented in the previous section indicate that Gale's conditions (pH 4·5; 30°), though satisfactory for the determination of glutamic acid, are not optimal for the decomposition of glutamine. A less acid medium of pH 4·9 and a temperature of 40° is therefore proposed. A pH higher than 5·0 would be disadvantageous with regard to the manometric determination of CO_2, because at such a pH the medium would retain CO_2. Furthermore, addition of cetavlon is recommended if the material to be examined does not contain some protein. Extracts of animal tissues usually accelerate the decarboxylation of glutamine sufficiently to make the addition of cetavlon unnecessary.

Reagents

Bacterial suspension. The stock suspension of *Cl. welchii* is diluted with 0·2 M-acetate buffer (pH 4·9) to contain *c.* 25 mg. dry bacteria/ml. If kept in a refrigerator the suspension may be used for about a month.

Stock acetate buffer. 160 ml. 3 M-Na acetate are mixed with 100 ml. 3 M-acetic acid. This buffer (pH 4·9; 3 M) is diluted to a final concentration not exceeding 0·2 M, since higher concentrations inhibit the enzyme.

Recovery of glutamine

Recovery of glutamine from pure solution was satisfactory as shown in Table 7. The errors in eight determinations did not exceed 4 % in the yield of CO_2 and 6 % in the yield of NH_3.

Table 7. *Recovery of glutamine from pure solutions*

(Procedure as described in text.)

Amount of glutamine analyzed (ml. 0·01 M-solution) ...	2	1	0·5	0·25
Glutamine expected (μl.) (allowing for 93% purity)	416	208	104	52
CO_2 (μl.) evolved: Duplicates	416	212	105	56
	419	212	103	50
Average	418	212	104	53
NH_3 (μl.) found: Duplicates	420	210	106	54
	400	216	110	50
Average	410	213	108	52

Table 8. *Changes in the concentration of glutamic acid and glutamine in animal tissues on storage*

(Procedure as described on p. 56 except that the tissue was not placed in liquid air; 'fresh' tissue was placed in iced water within a few minutes of death of the animal, and analyzed after being kept in the ice for about 50 min. 'Stored at 25°' refers to a sample of the same liver kept in ice for the same time and then left in a covered vessel at 25° for 24 hr.)

		Substance (μl./g. wet liver)		
		Glutamic acid	Glutamine	Preformed NH_3
Liver I:	Fresh	131	35	59
	Stored at 25°	225	14	386
Liver II:	Fresh	146	24	97
	Stored at 25°	305	15	305
Liver III:	Fresh	84	29	36
	Stored at 25°	152	5	278
Spleen:	Fresh	137	15	34
	Stored at 25°	179	~0	492

Specificity

The following substances, in quantities of 5 mg. each, did not produce NH_3 or CO_2 under the conditions of the glutamine determination:

Amino-acids: glycine, DL-alanine, DL-citrulline, L-arginine, L-phenylalanine, L-tyrosine, L-tryptophan, L-cysteine, L-cystine, DL-methionine, L-histidine, L-aspartic acid.

Acid amides: L-asparagine, urea, nicotinamide.

Purine derivatives: adenine, guanine, uric acid, allantoin, adenylic acid (muscle), adenylic acid (yeast), adenosinetriphosphate.

Miscellaneous: glucosamine, nicotinic acid, creatine, creatinine, succinimide.

Preparation of animal tissues for the determination of glutamic acid and glutamine

Preliminary experiments indicated that special precautions have to be taken when glutamic acid and glutamine are to be determined in animal tissues. The concentration of these two substances can undergo rapid changes, owing to the presence of proteolytic enzymes and glutaminase in these materials. Inactivation of the enzymes is not always practicable because operations commonly employed for this purpose are liable to destroy glutamine or to generate glutamic acid from protein or peptides.

When intact fresh tissue was kept at room temperature the glutamic acid increased and the glutamine fell (Table 8). In minced tissue, mixed with 2 parts of 0·1 N-HCl (resulting pH 3·7), both glutamic acid and glutamine rapidly increased even if the suspension was kept in the refrigerator (Table 9). The parallel increase in amino N indicates that the increase is due to proteolysis.

Table 9. *Changes in the concentration of glutamic acid and glutamine in acidified liver suspensions*

(Chilled sheep liver ground with 2 parts 0·1 N-HCl (pH of suspension 3·71 electrometrically). Amino N determined with nitrous acid method after deproteinization with trichloroacetic acid.)

	Substance (μl./g. wet wt.)			
Treatment of liver suspension	Glutamic acid	Glutamine	Preformed NH_3	Amino N
Fresh suspension	110	29	69	920
Stored 24 hr. near 0°	216	57	195	5,040
Stored 24 hr. at 25°	442	148	250	13,900

When 0·1 N-HCl was replaced by 0·3 N-HCl, the pH of the resulting tissue suspension being 2·2, an increase, though smaller, of both glutamic acid and glutamine was observed on 24 hr. storage in the refrigerator. On the other hand, the concentrations of glutamic acid and glutamine in a suspension containing 1 part of tissue and 2 parts of 0·5 N-HCl (pH about 1·0) remained almost constant in the refrigerator for 24 hr. After 48 hr., however, some of the glutamine had been converted into ammonium glutamate. The higher amount of

acid (0·5N) also inhibited the rise in 'preformed' NH_3, i.e. the NH_3 present in the tissue before the addition of *Cl. welchii* (Table 10).

Table 10. *Changes in the concentration of glutamic acid and glutamine in acidified liver suspensions*

(Rabbit liver frozen in liquid air for 20 min., thawed, minced and mixed with 2 vol. 0·5N-HCl.)

	Substance (μl./g. wet wt.)		
	Sum of glutamic acid and glutamine	Glutamine	Preformed NH_3
Examined immediately	138	27	50
Stored 24 hr. near 0°	138	28	48
Stored 48 hr. near 0°	139	12	61
Stored 24 hr. at 20°	152	—	—

A further error to be avoided is a loss of glutamic acid and glutamine, which occurs when tissue is packed in ice. The water from the melting ice penetrates into the tissue and causes it to swell. At the same time, amino-acids (and other substances) diffuse into the hypotonic medium. The result is that the concentrations of the amino-acids/unit weight of tissue fall. The magnitude of the changes is illustrated by the following example: 69 g. of liver, placed in 200 ml. water and 200 g. ice in a Dewar vessel, weighed 99 g. after 24 hr., whilst 52 mg. of glutamic acid had diffused into the water.

A complication interfering with the manometric CO_2 determination is the O_2 absorption which extracts or suspensions of liver, and to a less extent of testis, exhibit when shaken in air at pH 4–5. Most tissues tested (kidney, cortex and medulla, brain, spleen, heart, skeletal muscle, pancreas, thyroid gland) have no appreciable O_2 uptake under the experimental conditions. Sheep liver absorbed about 0·7–1·25 μl./mg. dry wt./hr. at 40°, without yielding CO_2. Often the rate increased with time. The substrate of this oxidation has not been identified. In some experiments the rate of the O_2 uptake increased on addition of the bacteria, and the tissue suspension alone is therefore not a satisfactory control to measure the O_2 uptake. The difficulties are overcome by filling the gas space of the manometers with N_2.

Procedure for the preparation of animal tissues. On the basis of the above observations the following procedure was adopted. The material, cut into 5–10 g. pieces, was thrown into liquid air as quickly as possible after death, and left there for at least 15 min. After removal from the Dewar vessel it was minced in a cooled tissue grinder as soon as the consistency permitted, transferred to a tared beaker, weighed, and 2 ml. of cooled 0·5N-HCl/g. tissue were added with thorough mixing. If the suspension could not be analyzed immediately it was stored in a refrigerator. For the determination of glutamic acid and glutamine, 2 ml. of suspension were pipetted into the main compartment of a conical Warburg vessel, followed by 0·1 ml. of 0·1 % aqueous methyl orange and just sufficient N-NaOH to change the indicator to yellow (usually about 0·6 ml.). Next, 0·2 ml. 3M-acetate buffer (pH 4·9) was added to the main compartment. The further procedure was as already described, except that the gas space of the vessels was filled with N_2 and the centre contained a stick of yellow phosphorus.

Recovery of glutamine added to tissue suspensions. When known amounts of glutamine were added to acidified liver suspensions recovery was satisfactory: for example, 73 μl. added, 74 μl. recovered; 36·5 μl. added, 41 μl. recovered.

Presence of inhibitors. Like other analytical methods based on the use of enzymes, the present procedure cannot be applied to solutions containing inhibitors of the enzymes concerned. If the presence of inhibitors in the unknown material cannot be excluded, glutamine should be added and its recovery tested. As the decarboxylation of glutamine is more sensitive towards some inhibitors than that of glutamic acid, the latter is not always a suitable test substrate. In pure solutions various indicators (0·01 %), such as phenolphthalein, bromocresol green, bromocresol purple and anionic detergents such as sodium dodecyl sulphate (0·05 %), were found to inhibit the decarboxylation of glutamine. These inhibitions were largely abolished by the addition of cetavlon (0·05 %) or serum (1 ml. to 3–4 ml. solutions), and the addition of one of these reagents may therefore be advisable if inhibitors are present. If serum is used, a blank determination is required; human serum contains about 8 mg. of glutamine and 2 mg. of glutamic acid/100 ml.

DISCUSSION

The main advantage of the present method over some previous techniques is the higher degree of specificity, coupled with relative simplicity. Vickery, Pucher, Clark, Chibnall & Westall (1935) deprecated the general application of their procedure (heating for 2 hr. at 100° at pH 6·5 and estimating the increase in NH_3-N) to unknown material because of lack of specificity. They reported that urea and allantoin yielded NH_3. Bartley & Krebs in unpublished experiments found that nicotinamide methochloride formed about 0·25 mol. of NH_3 when subjected to the procedure of Vickery *et al.* As nicotinamide methochloride is analogous to the pyridine nucleotides it is possible that these coenzymes also form NH_3. The specificity of Archibald's glutaminase from kidney is likewise imperfect, in that it is contaminated with deaminases liberating NH_3 from amino-purine nucleotides.

It cannot be claimed that the specificity of the glutaminase of *Cl. welchii* (strain S.R. 12) is absolute. However, it seems to be very high, since so far no substance apart from glutamine has been found to form NH_3 under the experimental conditions. Moreover, in a large number of analyses of animal and plant tissues, no material was encountered where the yield of NH_3 exceeded that of CO_2. If substances other than glutamine can produce NH_3 with the bacterial enzyme, an excess of NH_3 over CO_2 might have been expected.

SUMMARY

1. L-Glutamine yields one molecule each of NH_3 and of CO_2 when added to washed suspension of *Clostridium welchii* in acid solution. The formation of NH_3 precedes that of CO_2. The 'glutaminase' of the organism is highly specific; none of many substances tested was found to yield NH_3.

2. The method for the determination of glutamine and glutamic acid combines the principles used by Gale and by Archibald. The sum of the substances is determined according to Gale by manometric determination of the CO_2 evolved on decarboxylation. The NH_3 is then estimated in the solution treated with *Cl. welchii* and in a sample not treated with the bacteria. The difference represents the glutamine.

3. The conditions affecting the quantitative reaction of glutamine were examined. In pure solutions glutamine reacted much more slowly than glutamic acid, and the rate of reaction of glutamine, and to a smaller extent that of glutamic acid, was much (in some cases more than tenfold) accelerated by cetyltrimethylammonium bromide, serum or tissue extracts. In the presence of these substances the maximum rates of the decarboxylation of glutamine and glutamic acid were approximately equal.

4. The slow reaction of glutamine seems to be due, under some conditions, not to the low activity of the glutaminase or glutamic decarboxylase, but to permeability barriers. Removal of these barriers would account for the accelerating effect of cetyltrimethylammonium bromide.

5. The glutamine and glutamic acid content of animal tissues rapidly increases after death owing to autolysis. A procedure has been elaborated which minimizes the effects of autolytic enzymes.

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The Recovery of *l*-Nicotine from Animal Tissues and its Colorimetric Micro-estimation

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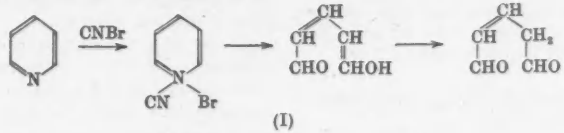
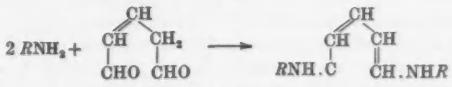
Some heterocyclic substances containing a trivalent nitrogen atom react with 1:2:4-chlorodinitrobenzene (Vongerichten, 1899; Reitzenstein, 1903), cyanogen bromide, phosphorus pentachloride (König, 1904) and other substances to produce an unstable pentavalent nitrogen derivative which immediately changes to an open chain derivative of glutaconic aldehyde (see (I)). A similar change occurs in the action of cyanogen bromide on furfural, when the ring opens at the cyclic oxygen atom. The glutaconic derivative obtained in this way will condense with certain aromatic amines to form stable coloured compounds (see (II)). The tint and intensity of colour produced depend primarily upon the structure of the heterocyclic base and the amine. In the cases which have been investigated



(I)



(II)

the development of colour is also influenced by the pH and the concentration of inorganic ions in the solution.

This reaction has been widely used for the detection and estimation of pyridine derivatives of biological importance. The procedures adopted have been reviewed by Bacharach (1941) and Waisman & Elvehjem (1941). Barta & Marschek (1937) estimated nicotine in tobacco by measuring the colour produced by the action of cyanogen bromide and β-naphthylamine on extracts of the plant. This procedure has now been developed for the accurate estimation of nicotine recovered from animal tissues by a distillation procedure. Quantities up to 600 μg. of the base were recovered from 2 to 10 g. wet weight of tissues in 15 ml. of distillate. Quantities of 2 μg./ml. distillate and upwards were estimated with an error of ± 1 %.

EXPERIMENTAL

Reagents. (1) *l*-Nicotine, purified by distillation under reduced pressure, b.p. 122–123°/16 mm., n^{20} 1·5230. Standard solutions were prepared by dissolving a weighed quantity of freshly distilled nicotine in water immediately before use.

(2) Cyanogen bromide, prepared in aqueous solution by the method of Larson & Haag (1944). This was only necessary for the estimation of small quantities of nicotine with maximum accuracy, and for most purposes the solution obtained by just decolorizing saturated bromine water with 10 % (w/v) KCN solution was found to be suitable. This preparation was diluted five times with distilled water and adjusted to pH 6 with KCN solution. If kept at 0° both solutions lasted for a week without deterioration.

(3) Ethanolic β-naphthylamine. β-Naphthylamine was recrystallized from ethanol immediately before use to remove the coloured substances which are rapidly formed from it on exposure to light. The purified amine was made up in 0·2 % (w/v) solution in 95 % ethanol before use.

(4) 0·2M-Sorensen's phosphate buffer, pH 5·9–6·1.

(5) Acid aqueous ethanol. This consisted of a mixture containing 9 parts by volume 50 % ethanol and 1 part of a mixture of equal volumes of glacial acetic acid, concentrated HCl and amyl alcohol.

The reagents were used in the following order and proportions: nicotine solution, 1·0 ml.; buffer, 0·2 ml.; CNBr solution, 0·27 ml.; ethanolic β-naphthylamine, 1·0 ml.

Factors influencing the rate of colour development and intensity

A number of workers (see Bacharach, 1941; Waisman & Elvehjem, 1941) have used this reaction for the estimation of nicotinic acid and nicotinamide. The more recent investigations have shown that the intensity of colour developed is markedly influenced by the pH in the solution and by the presence of inorganic ions. This was also found to be the case with *l*-nicotine. The addition of the phosphate buffer indicated above led to the development of the maximal intensity of colour. This buffer was the sole source of inorganic ions present in the first stage of the estimation.

The rate of colour development, which is slow at room temperature, was increased by heating to 37°. At 37° and the optimum pH 5·9–6·1 it was found that the colour developed was yellow which gradually increased in intensity and changed to orange red. Measurements with a spectrophotometer showed that there is a peak of extinction between the wave lengths of 490 and 550 mμ. In view of this, green colour filters were used in subsequent investigations. Measurements were made with the Spekker photoelectric absorptiometer, using the green filter no. 5, and in routine visual colorimetry with the Duboscq type colorimeter Chance's glass filter 0 Gr. 1 was employed. With the aid of the absorptiometer it was found that the intensity of colour reached a maximum in 2·5 hr. and remained constant up to 20 hr. A typical set of results is shown in Fig. 1. A series of tubes containing 300 μg.

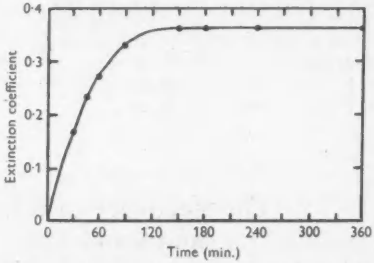


Fig. 1. Nicotine (300 μg.), cyanogen bromide and ethanolic β-naphthylamine. Relation between extinction coefficient and the time of heating at 37°.

of nicotine in 1 ml. of solution was treated with appropriate volumes of reagents and kept at 37°. At intervals tubes were removed in duplicate, the contents made up to 20 ml. with 50 % ethanol and the optical density read in the absorptiometer. The relation between the extinction coefficient and the time of heating is shown.

If one of the above solutions is taken after a minimum of 2·5 hr. heating, made up to 20 ml. with 50 % ethanol and allowed to stand in the air, the second colour change takes place. A slow increase of optical density occurs and the tint changes towards magenta. This change is greatly accelerated by the addition of acid aqueous ethanol (reagent 5) in the place of 50 % ethanol. Fig. 2 shows the relation between the extinction coefficient and the time of keeping at room temperature of solutions from the above series which had been heated for 2·5 hr. at 37° and then made up to 20 ml. with acid aqueous ethanol. The diagram shows that the change is complete within 2 hr. However, in practice it is

not necessary to wait for 2 hr. before reading, for the relative rate of increase of extinction is independent of the original concentration of nicotine. Provided, therefore, that the acidified ethanol is added to all the solutions at the same time, they may be compared with a standard, prepared simultaneously with them, at any time after the addition. Absolute readings with the absorptiometer can, of course, only be taken after the full 2 hr. has elapsed.

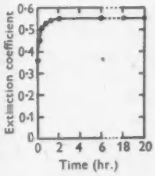


Fig. 2.

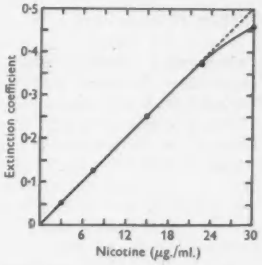


Fig. 3.

Fig. 2. Nicotine (300 μg.), cyanogen bromide and ethanolic β-naphthylamine. Relation between extinction coefficient and the time of keeping with acid ethanol at room temperature after the full development of the primary colour at 37° and pH 6.

Fig. 3. Relation between the extinction coefficient of the reaction mixture and the concentration of nicotine.

Beer's law. As is shown by Fig. 3, there is a linear relationship between colour and nicotine concentration over a wide range (in this case 1·5–20 μg./ml.).

The estimation of micro-quantities of nicotine

It is clear that, if it is contained in a suitably small volume, about 1 μg. of nicotine may be estimated by this method, using either a direct-vision colorimeter with small cups, capacity *c.* 2 ml., or a photoelectric colorimeter with 2 ml. cuvettes, with suitable colour filters. The error is less than 5 %.

As will be seen in a later section, the minimal volume in which all the nicotine may be recovered from animal tissues is 15 ml., so that when very small amounts of nicotine are present it may be necessary to concentrate the solution. This may be done by reducing the pH to 4 with HCl and evaporating under reduced pressure.

The recovery of nicotine from tissues

Nicotine is recovered from tissues by taking advantage of its volatility in steam. The nature of the tissues under investigation and the use to be made of the distillate will determine the precise procedure employed. For the purpose of colorimetry it is desirable to have the nicotine in a minimum volume of distillate. It was found that the base could be recovered quantitatively from the tissues of the nematode *Ascaris lumbricoides* var. *suis* by a modification of the procedure of Werle & Becker (1942). The distillation apparatus, which is based on the Pregl micro-Kjeldahl apparatus, is illustrated in Fig. 4. Fresh worm tissue (2–10 g.), containing nicotine, was chopped into the detachable flask *A*. The following reagents were then added: (1) distilled water to a total weight of 25 g., (2) 11 g. of NaCl, and (3) 0·3 g. of MgO.

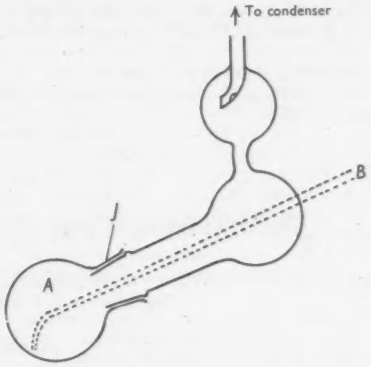


Fig. 4. Diagram of distillation apparatus for the recovery of nicotine from tissues.

Table 1. *Recovery of nicotine from tissues of* Ascaris

l-Nicotine added (μg.)	Wet wt. tissues (g.)	Recovery of nicotine (%)
30	5·7	100
30	6·4	100
60	6·7	99
60	5·1	100
150	6·0	100
300	5·0	98
300	5·2	100
600	5·5	97
600	5·5	99

The flask was attached to the apparatus by the ground-glass joint *J* and supported by a ring and gauze. The mixture was then heated by a small gas flame, and 15 ml. of distillate were collected, care being taken to avoid frothing beyond the first bulb of the still head. Some examples of the recovery of nicotine are given in Table 1. In this experiment small volumes of nicotine solution, *c.* 1 ml., were added to chopped *Ascaris* tissues, incubated at 37° for 6 hr., and then distilled. The final volume of solution for estimation was 50 ml.

The process of distillation was slow, taking about 30 min. for the complete operation. Recoveries adequate for exploratory and comparative work may be obtained by a much more rapid steam distillation procedure in which the flow of vapour is supplemented by the passage of steam through the tube *B* (broken line, Fig. 4). By this procedure about 75% of the nicotine may be recovered in 5 min. in 15 ml. of distillate.

SUMMARY

A method is described for the recovery of nicotine from tissues and its estimation in quantities from 1 μg.

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Decomposition and Synthesis of Cozymase by Bacteria

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Investigations concerning the metabolism of nicotinic acid and its derivatives have been surveyed recently by Schlenk (1945), and, in the case of micro-organisms, by McIlwain (1947). In the latter account, attention was directed to the synthesis, inactivation and interconversion of coenzymes I and II by micro-organisms, and particular efforts were made to obtain values for the rates at which these changes occur. Although many papers bearing on this subject were assessed, few if any were found to contain all the data required for calculating unequivocally the rates at which these important processes take place. On the other hand, such estimates of the rates as could be made by supplying likely values for missing data suggested the existence of a group of reactions with velocities of a few mμmol./mg. dry wt. of organism/hr. The possible significance in bacteria of reactions of this magnitude has been discussed elsewhere (McIlwain, 1946*b*).

Studies have now been made of the behaviour of bacteria towards nicotinic acid derivatives, especially cozymase. This behaviour has been found to be dominated in many organisms by reactions which are potentially much more rapid than those inferred previously. Before studying the slower reactions (for which, also, evidence has been obtained) the course and products of the more rapid reactions have been examined, and are reported in this paper.

EXPERIMENTAL

Organisms. A β-haemolytic streptococcus (R, the Richards strain; National Collection of Type Cultures no. 5631), two strains of *Streptococcus faecalis* (F 4208, N.C.T.C. no. 4208; FL, a laboratory strain), a *Staphylococcus aureus* (laboratory strain) and *Proteus morganii* (N.C.T.C. no. 2818) were grown in the casein-yeast medium of McIlwain (1946*a*), modified as follows: in place of NaOH, 0·5M-$NaHCO_3$ (additional 6·8 ml./100 ml.) was added; the initial pantothenate content was 10^{-6}M; the quantity of yeast preparation was 2·5ml./100 ml.; and of the group A addenda only riboflavin, aneurin, KH_2PO_4, tryptophan, methionine, $MgSO_4$, $Fe(NH_4)_2(SO_4)_2.6H_2O$ and cystine were included. *Escherichia coli* (N.C.T.C. no. 4074) was grown in the mixture of inorganic salts and glucose of Dorfmann & Koser (1942).

Lactobacillus arabinosus 17–5 was grown in a medium elaborated for the use of the organism in assaying nicotinic acid (see below), and containing the quantities of nicotinic acid specified in the individual experiments described. Its pH fell from 6·8 to 5 during growth. *Haemophilus parainfluenzae* was also grown in the corresponding assay medium, with defined quantities of cozymase as V-factor.

Reaction with cozymase. Bacteria were normally harvested by centrifuging, washed twice with 0·9% NaCl, suspended in that solution and a small sample taken for dry weight estimation. The suspensions were distributed between experimental vessels, which, for anaerobic experiments, were Warburg flasks with yellow P in a centre well. Saline was that of Krebs & Henseleit (1932).

Microbiological assays. Nicotinic acid was determined using *Lactobacillus arabinosus* 17–5 in very nearly the way

described by Barton-Wright (1946) (see McIlwain & Stanley, 1948). V-factor was estimated by growth of *Haemophilus parainfluenzae* (N.C.T.C. no. 4101) in a medium similar to that of Hoagland & Ward (1942); growth, however, was measured photoelectrically. Cozymase in concentrations of $0{\cdot}3–2 \times 10^{-8}$ M was used as source of V-factor in obtaining reference curves, and results are expressed in terms of cozymase. The inoculum was 0·05 ml. of a culture grown for 24 hr. in the assay medium with 10^{-7} M-cozymase. Inoculated cultures of 10 ml. final volume were incubated in 50 ml. conical flasks at 37° for 30–48 hr.

Determination of cozymase using apozymase. Though the determination of cozymase using apozymase (Euler, 1936; Myrbäck, 1933) requires care, the method appears much more reliable than was suggested by Jandorf, Klemperer & Hastings (1941). By adopting precautions specified by Axelrod & Elvehjem (1939) and others noted below, we have consistently obtained reliable results. Our experience and practice have been in some cases similar to, and, in others, different from that of Schlenk & Schlenk (1947), whose paper appeared while the present one was in preparation.

Preparation of apozymase. Fresh brewer's yeast was dried in air and then over $CaCl_2$; it can be kept in this form for some months. The washing, drying, rewashing and redrying (all these stages were found necessary) to yield apozymase were carried out according to Axelrod & Elvehjem (1939) and in one day. The product was kept in a stoppered bottle over $CaCl_2$ in a refrigerator for periods up to a fortnight.

It was found necessary to carry out the preparation of apozymase at room temperature (15–20°). When, in trying to obtain a more stable preparation, washing was performed with ice water, the apozymase obtained was deficient in cozymase, but its response to certain solutions containing cozymase increased with time. Such behaviour has been observed by Euler & Adler (1938), Euler, Adler & Eriksen (1937) and by Lennerstrand (1941), and interpreted as due to synthesis of cozymase. We have not found such behaviour in apozymase prepared as described in the preceding paragraph, and have therefore not found it necessary to adopt a method such as that of Lennerstrand's (1941) 'pyocyanin-system', which is much less sensitive to cozymase than is the normal apozymase system.

Preparation of hexosediphosphate. Neuberg's (1942) method was followed, using fresh baker's yeast; fermentation was complete in 5 hr. and the yield of calcium hexosediphosphate from 200 g. of sucrose was *c.* 16–20 g. A solution of sodium hexosediphosphate was prepared freshly each day; the Ca salt (208 mg.) was dissolved in 0·49 ml. of ice-cold N-HCl and the theoretical quantity of sodium oxalate, in 3·5 ml. water, added gradually. The pH was brought to 7 by N-NaOH (0·4 ml.), the mixture left cold for 10 min. and centrifuged. The solution was taken to pH 6·2 with N-HCl (*c.* 0·15 ml.) for use in the apozymase system; it contained *c.* 4 mg. organic P/ml.

Cozymase determination. This followed Axelrod & Elvehjem's (1939) description, but was performed in Warburg vessels with one side arm and of total volume *c.* 18 ml. With the apozymase used, response of the system at 30° to cozymase was found to be maximal with phosphate concentrations between 0·055 and 0·07 M, and the quantity of phosphate buffer added for cozymase determination was such as to give a final concentration of 0·065 M. This concentration is higher than that adopted by Axelrod & Elvehjem (1939), but other reagents were used in concentrations described as typical by those authors. If the solutions whose cozymase content was being determined were significantly buffered, they were brought to pH 6·2, and if their phosphate content was more than 0·02 M, that of the remainder of the system was reduced accordingly. With 0·2 g. of apozymase/vessel, the system was used for determination of up to 15 or 20 mμmol. of cozymase, and standard solutions of 7·5 and 15 mμmol. of cozymase were included with each batch of unknown solutions. It is necessary to avoid citrate buffers in solutions whose cozymase is being determined by apozymase.

Other determinations of cozymase were made by dismutation of hexosediphosphate (method and muscle preparation of Jandorf *et al.* 1941) and reaction with hyposulphite, following Warburg & Christian's (1936) description.

Cozymase specimens. Pure cozymase has not been obtained, and unless otherwise stated, specimen A of Table 1 has been used, but its behaviour has been compared with two independent specimens B and C of Table 1. The different methods of estimating the cozymase content of specimen A, quoted in Table 1, gave values differing by not more than 6%, and their mean value of 40% purity has been adopted in interpreting results with this specimen. The principles underlying methods I and II are entirely different from each other and from that underlying methods III and IV. The approximate agreement between methods I and II suggests that specimen A contains practically all its nicotinic acid as a quaternary salt such as cozymase. The approximate

Table 1. *Analytical data concerning cozymase specimens*

	Determination and standard value	Cozymase specimen A	B	C
I	Acid formation in reaction with $Na_2S_2O_4$ (μmol./mg.)	1·75	0·50	1·80
	Purity by comparison with theoretical value of 4·5 μmol./mg. ($C_{21}H_{27}O_{14}N_7P_2$, mol.wt. 663) (%)	37	11	40
II	Nicotinic acid content (%)	8·0	—	—
	Purity (theoretical value 18·6%) (%)	43	—	—
III	Catalytic activity in apozymase system (μmol. CO_2/μg./hr.) (S.D. of 15 results, with specimen A, 0·18)	1·47	0·44	1·75
	Purity (by comparison with value of 4·0 derived from Axelrod & Elvehjem, 1939) (%)	37	11	44
IV	Catalytic activity in Jandorf *et al.* (1941) system (μmol. CO_2/μg./hr.)	0·89	—	—
	Purity (by comparison with value of 2·15, derived from Jandorf *et al.* 1941) (%)	41	—	—

agreement between methods II, III and IV implies that the specimen contains relatively little material containing nicotinic acid which is not cozymase. The agreement between method III and the other methods justifies our dependence on method III in most of the subsequent work. We have also shown (Table 7) that the nicotinic acid grouping of specimen A exists as its amide, without detectable free acid (cf. Schlenk, 1945).

Extraction of bacterial cozymase. Extractions were performed aerobically in phosphate buffer, pH 6·2, as this is the medium in which cozymase is estimated in the apozymase system, and pH 6·2 is close to that of optimal stability of cozymase. Previous investigators have heated for short periods at temperatures of 70–100° in order to obtain cozymase from natural materials, and we have examined the yield of cozymase from streptococci under such conditions. Results quoted in Table 2 show that heat treatment for 8–30 min. at these temperatures yields extracts containing about the same quantity of cozymase, but that 15 min. at 70° is optimal. The quantity of cozymase so obtained was found to be nearly the same as that extracted from the cells by grinding with glass. Heating at 70° for 15 min. has therefore been employed throughout the studies reported in this paper for extracting cozymase; Table 2 shows that 5% of the substance may be lost under these conditions, but values quoted have not been corrected for such loss.

RESULTS

Inactivation of cozymase

Behaviour of various bacterial species. Washed suspensions of bacteria were incubated in buffered salt solutions containing 10^{-5}–10^{-4} M-cozymase. The majority of species inactivated cozymase (Table 3). Reaction at the rate of 5–30 mμmol./mg./hr. was found in β-haemolytic streptococci, *Haemophilus parainfluenzae*, *Staphylococcus aureus* and *Proteus morganii*. *Escherichia coli* and *Streptococcus faecalis* were less active; *Lactobacillus arabinosus* 17–5 was the only organism of those examined with activity $<0{\cdot}5$ mμmol./mg./hr.

Circumstances affecting the reaction. Most of the following investigations were with streptococci. The reaction reached its greatest velocity at a pH near 6 or 7 (Table 4; see also Table 8). We have usually studied it at pH 7–7·5 in order to assess its relation to growth. With high concentrations of cozymase, very rapid rates of inactivation were reached by all three species examined. In Table 5, cozymase at an initial concentration of $1{\cdot}2 \times 10^{-3}$ M is seen to be

Table 2. *Extraction and stability of cozymase from streptococci*

(Streptococci throughout were the Richards strain, grown in the casein yeast medium for (in different batches) 20–40 hr.)

Material extracted or treated	Conditions of extraction or treatment	Cozymase		
Streptococci, batch A	At 70°, 15 min.; 2·4 mg. cells/ml.	6·3		mμmol./mg. cells
	At 70°, 30 min.; 2·4 mg. cells/ml.	5·9		
	At 85°, 15 min.; 2·4 mg. cells/ml.	5·8		
		(Batch B)	(Batch C)	
Streptococci, batches B and C	At 70°, 15 min.	9·8	4·9	
	At 85°, 15 min.	9·0	4·3	
	At 100°, 8 min.	8·5	3·8	
Streptococci, batch B	Ground with glass	9·5		
	Ground with glass and extract heated at 70°, 15 min.	9·2		
Streptococcal extract	None	(*a*)		
	Heated at 70°, 15 min.	94% of (*a*)		
Cozymase	None	(*b*)		
	Heated at 70°, 15 min.	94–97% of (*b*)		
Streptococci with added cozymase:				
Cells (4·8 mg.)	At 70°, 15 min.	48		mμmol.
Cozymase	At 70°, 15 min.	82		
Cozymase with cells (4·8 mg.)	At 70°, 15 min.	126		

The rapid inactivation of cozymase by streptococci makes it possible that losses may occur by enzymic activity during the heating to 70°, but we have not found this to be the case. The extraction has been carried out in small volumes of liquid (usually <2 ml.) placed in tubes in a water bath at 70°. Temperatures approaching 70° are quickly attained in the tube, and cozymase added to the system is recovered satisfactorily (Table 2).

inactivated at over 440 mμmol./mg./hr. The system does not approach saturation even at that concentration; values for $(\log a/b)/t$ (a = initial, b = concentration at time t) are not falling at the higher concentrations of Table 5A. In Table 5B, also, the velocity is proportional to the initial concentration of cozymase, except with the lower concentration

Table 3. *Bacterial inactivation of cozymase*

(Reactions were at 37°, with shaking, in tubes or Warburg vessels. The latter, with yellow P in a centre well and N_2 in the gas space, were employed for anaerobic reactions. Changes in cozymase refer to the whole reaction mixture including the cozymase of the cells.)

Reaction mixture			Reaction performed aerobically (A) or anaerobically (AN)	Rate of change in cozymase (mμmol./mg./hr.)
Organism and dry wt. of cells/ml.	Cozymase (M × 10^{-5})	Other constituents		
β-Haemolytic streptococcus (R); 3·9 mg./ml.	2·4	0·02 M-Na and K phosphates, pH 7·6	A	−29
Streptococcus faecalis (FL); 3 mg./ml.	1·2	0·03 M-Na and K phosphates, pH 7·6, with saline	AN	−1·8
	1·2	Do.	A	−1·7
Staphylococcus aureus (E); 3·2 mg./ml.	1·2	Do.	AN	−7·0
	1·2	Do.	A	−5·2
Proteus morganii (2818); 1·3 mg./ml.	1·2	Do.	A	−4·2
	1·2	Do.	AN	−4·5
Lactobacillus arabinosus 17–5; 21·5 and 45 hr. old; 2·3 mg./ml.	6	As above; also with glucose (0·05 M)	A	<0·5
Lactobacillus arabinosus 17–5; 40 and 44 hr. old; 3·6 mg./ml.	6	As above; pH 5 and 7	A	<0·5
Haemophilus parainfluenzae, N.C.T.C. 4101; *c.* 2 mg./ml.	6	0·03 M-Na and K phosphates, with saline; pH 6 and 7	A	−6·7 to 8·4
Escherichia coli; 7 mg./ml.	2·4	As above at pH 7·5	A	−0·6
	2·4	Do.	AN	−3·5
	2·4	As above with glucose (0·02 M)	AN	−1·1

Table 4. *Cozymase inactivation by streptococci: different specimens*, pH, *concentrations and temperatures*

(Reactions were aerobic and in phosphate saline, except * which was in acetate buffer. Cozymase specimens: see Table 1.)

Cozymase					
Specimen	Initial concentration (M)	Organism (batch)	Temp.	pH	Rate of inactivation (mμmol./mg./hr.)
A	4×10^{-4}	R (*a*)	10	7·5	172
A	4×10^{-4}	R (*a*)	20	7·5	267
A	4×10^{-4}	R (*a*)	37	7·5	390
A	4×10^{-4}	R (*b*)	37	7·5	295
C	$3·6 \times 10^{-4}$	R (*c*)	37	7·5	160
C	$3·6 \times 10^{-4}$	R (*d*)	37	7·5	266
B	$3·6 \times 10^{-4}$	R (*d*)	37	7·5	250
A	$1·2 \times 10^{-3}$	FL	37	7·5	288
A	$3·6 \times 10^{-4}$	F 4208	37	7·5	144
A	4×10^{-4}	R (*e*)	37	5*	37
A	4×10^{-4}	R (*e*)	37	6	183
A	4×10^{-4}	R (*e*)	37	7	159
A	4×10^{-4}	R (*e*)	37	8	84

when measurements were less accurate. Different cozymase specimens and different streptococcal strains behaved similarly, and the reaction was still rapid at 10° (Table 4).

The course of change in cozymase in one reaction mixture was followed both by apozymase and by the muscle preparation of Jandorf *et al.* (1941). Results obtained by the two methods did not differ by more than 6%.

Effects of other substances on the reaction. Inactivation of cozymase in the presence and absence of oxygen is compared with several organisms in Tables 3 and 6. Differences between the two conditions were in general not striking and often within experimental error. Any difference was in the sense of greater loss anaerobically; the greatest contrast was found with a *Streptococcus faecalis* (Table 6) and *Escherichia coli* (Table 3).

Table 5. *Course of the streptococcal inactivation of cozymase*

(In Exp. A, organisms of strain R were grown in 500 ml. of standard medium, collected, washed twice in 0·5% NaCl and the bulk, of dry wt. 190 mg., suspended in 10 ml. of 0·5% NaCl and mixed with cozymase (33 μmol.) in 10 ml. of 0·04 M-Na and K phosphates, pH 7·6. The mixture was shaken at 37° in air, and specimens (0·2 ml.) were taken at the intervals indicated, diluted in cold water, and their cozymase determined in the apozymase system.

For Exp. B, a smaller quantity of a similar reaction mixture was serially diluted to give the four different concentrations of cozymase (and of organisms) which are indicated.)

Reaction period (min.)	Cozymase content: μmol. in whole	Cozymase content: M × 10^{-4} (*c*)	Mean $Q_{Coz.}$ (mμmol./mg./hr.)	Log *a*/*b* / Time (hr.)
		Exp. A		
0	33·3 (*a*)	16·7	—	—
20	13·5 (b_1)	6·75	−155	1·18
40	6·04 (b_2)	3·02	−117	1·11
60	2·28 (b_3)	1·24	− 59·4	1·16
90	1·22 (b_4)	0·61	− 16·7	0·96
		Exp. B		$Q_{Coz.}/c$
15	—	12	−444	−37
15	—	4	−140	−35
30	—	1·33	− 48	−36
60	—	0·44	− 11	−25

Table 6. *Distribution of cozymase between bacterial cells and suspending fluid during its inactivation*

(Reactions were carried out in 2·5 ml. of phosphate saline, pH 7·5.)

Reaction mixture: Organisms (mg. dry wt.)	Other reagents	Added cozymase (mμmol.)	Reaction: Aerobic (A) or anaerobic (AN)	Time (hr.)	Cozymase (μmol.): In cells	In solution	Total	Loss
			Streptococcus faecalis					
8·4	—	—	A	2	43	4	47	—
8·4	—	62	A	2	60	35	95	14
8·4	—	62	AN	2	49	33	82	27
8·4	—	62	AN	0·5	54	48	102	7
8·4	Glucose; 0·01 M	62	AN	0·5	61	38	99	10
			Staphylococcus aureus					
9·5	—	—	AN	2	53	< 1	53	—
9·5	—	30	AN	0·5	54	< 1	54	29
9·5	Glucose; 0·01 M	30	AN	0·5	48	< 1	48	35
9·5	—	30	A	2	53	7	60	23
			Proteus morganii					
3·7	—	—	AN	2	41	3	44	—
3·7	—	34	AN	2	37	3	40	38
3·7	—	34	A	2	38	2	40	38
			Escherichia coli 4074					
7·4	—	—	—	—	26	—	26	—
7·4	—	93	AN	0·5	42	13	55	64
7·4	—	93	AN	2·0	40	< 1	40	79
7·4	Glucose; 0·01 M	93	AN	0·5	58	14	72	47
7·4	—	93	A	2·0	56	1	57	62

Glucose did not have a large effect on the reaction; instances of greater loss in its presence are shown in Table 6 with streptococci and staphylococci. During these experiments both organisms caused a rapid breakdown of the added glucose, largely to lactic acid. Glucose, on the other hand, decreased the inactivation of cozymase brought about by *Esch. coli*. Sodium pyruvate, α-ketoglutarate, and glutamine (all at 7×10^{-3} M) were not found to alter the rate of change in cozymase added anaerobically to suspensions of the R streptococci. Variation in the phosphate concentration of the medium between 0·008 and 0·08 M caused no change in the reaction with cozymase.

Distribution of cozymase between cells and solution. The experiments of Tables 3–5 concerned reaction mixtures in which the added cozymase was in large excess over that of the bacteria, or in which the cozymase of the complete reaction mixture had been determined after liberating the substance from the cells. The experiments of Table 6 were performed with relatively little added cozymase, and the distribution of cozymase between the bacteria and the solution was examined. Added cozymase was found first to enter the cells and then to be lost without reappearing in solution. Much added cozymase could be lost with relatively little change in the cellular cozymase. Almost all the nicotinic acid derivatives resulting from the breakdown of cozymase in Exp. A, Table 7, were found in solution and not in the cells.

Products of cozymase inactivation by streptococci

Nicotinamide moiety. Although the specimens of cozymase which were employed in this investigation were not pure, they contained little nicotinic acid which was not in the form of cozymase (Table 1). The bulk of the nicotinic acid of streptococci also appeared to exist as cozymase (Table 7C). When streptococci were caused to react with added cozymase, the cozymase content of the system fell without a corresponding decrease in its content of nicotinic acid derivatives. This is shown in each experiment of Table 7.

The nicotinic acid estimations of Table 7 were performed after heating with acid under conditions (see experimental section) which other workers have found to liberate nicotinic acid from the majority of its naturally occurring derivatives. The preceding observations, therefore, do not imply that nicotinic acid itself is formed from cozymase. As nicotinic acid exists in cozymase as its amide, we have sought information on the fate of the amide grouping. Atkin, Schultz, Williams & Frey's (1943) method for distinguishing between nicotinamide and nicotinic acid was found to be an excellent one, and applicable when nicotinic acid was assayed with *Lactobacillus arabinosus*. Solutions were assayed before and after treatment with hypobromite. This converted the amide but not the acid to the amine, which was without growth-promoting activity. Table 7B shows the product from cozymase to behave similarly to nicotinamide in this respect. It also shows the specimen of cozymase which we have employed to contain $<0{\cdot}5\%$ of free nicotinic acid, or of derivatives of nicotinic acid which failed to be inactivated by hypobromite (cf. Schlenk, 1945).

The stability of nicotinamide, when added as such to streptococci, was investigated to see whether it was comparable to that of the product from cozymase. Reaction mixtures similar to that of Exp. A, Table 5, but containing nicotinamide in place of cozymase, were kept at 37° for 90 min. Ammonia was determined by Nessler's reagent after distillation under reduced pressure. Ammonia formation occurred in the absence of nicotinamide at the rate of about 40 mμmol./mg./hr., and no difference was detected in this rate when 4 or 40 μmol. of nicotinamide were added. A difference of 10 mμmol./mg./hr. could have been detected. Corresponding quantities of cozymase would have been inactivated at rates of at least 400 mμmol./mg./hr. under the conditions of the reaction. Any hydrolysis of nicotinamide thus appears to be at a rate not exceeding 10% of that of the inactivation of cozymase; in reality, it may be much less.

Loss of V-factor activity. The term V-factor has been retained as a collective name for a group of

Table 7. *Stability of nicotinamide moiety of cozymase in the presence of streptococci*

(In mixtures A, varying quantities of streptococci R, prepared as described in Table 5, were incubated aerobically with cozymase in 0·017 M-Na and K phosphates of pH 7·5. At the times indicated, portions of the reaction mixtures were cooled, centrifuged and the cozymase (by apozymase) and nicotinic acid in solution were determined. Mixtures B were similar but determinations were carried out on the whole reaction mixture, including bacteria. Mixtures C were buffered with veronal (0·05 M, pH 7·5) and determinations included bacteria.)

	Mixture	Cozymase content (mμmol.)	Nicotinic acid content (mμmol.)	Nicotinic acid remaining after treatment with hypobromite (mμmol.)
A.	Before reaction	57·3	55	—
	With 11·4 mg. organisms, 10 min.	3·9	57	—
	With 11·4 mg. organisms, 40 min.	3·6	60	—
	With 3·8 mg. organisms, 10 min.	23·7	59	—
	With 3·8 mg. organisms, 40 min.	11·7	61	—
B.	Organisms and cozymase before reaction	24	26	0·1
	Organisms and cozymase after reaction	0·4	27	0·15
	Nicotinic acid	—	406	420
	Nicotinamide	—	406	1·2
C.	Organisms and buffer	60	68	—
	Cozymase and buffer	102	115	—
	Above quantities of organisms, cozymase and buffer after 1 hr. at 37°	96	172	—

substances, any one of which will support the growth of *Haemophilus parainfluenzae* under certain conditions described in the experimental section. These substances are known to include coenzymes I and II, a 'desaminocozymase' (Schlenk, Hellström & Euler, 1938) and nicotinamide riboside. The simplest of these is the riboside, and the others are derivatives of it.

When cozymase had been inactivated with respect to the apozymase or muscle systems, we found that it had lost also most of its V-factor activity. Moreover, when part only of its activity in the apozymase system had been lost, a similar part of its V-factor activity had disappeared (Table 8).

Isolation of nicotinamide picrolonate. The simplest conclusion from the preceding paragraphs is that the streptococcal reaction with cozymase produces a molecule smaller than nicotinamide riboside, but still containing the nicotinamide part of the molecule. Following a method similar to that of Handler & Klein (1942), who were concerned with the breakdown of cozymase by animal tissues, we have attempted to isolate nicotinamide itself from the products of streptococcal inactivation of cozymase.

Table 7 shows that the product from added cozymase which carries the nicotinic acid portion of the molecule remains in solution and not associated with the streptococci. The reaction mixture of Table 5A was accordingly kept at 37° until samples showed all but 3% of its cozymase to be inactivated. It was then centrifuged and the organisms washed twice with 8 ml. of water. The combined solutions were acidified to pH 3 with 5 N-H_2SO_4, and Ag_2SO_4 (1·7 mmol). added until no more precipitate formed; this was separated. The solution was freed from Ag^+ by H_2S, and after removing the latter by N_2, it was taken to pH 9 with $Ba(OH)_2$, N_2 again passed, then adjusted to pH 7–7·5 with H_2SO_4, $BaSO_4$ removed and washed, the combined solutions evaporated to dryness under reduced pressure and the residue left *in vacuo* with $CaCl_2$ overnight. It was extracted by refluxing 3 times successively with 8 ml. of absolute ethanol, the ethanol solutions evaporated to dryness and the residue re-extracted 3 times with 2 ml. of absolute ethanol. Determination of nicotinic acid showed that all the acid had not been extracted by these procedures; the residues were accordingly re-extracted in the same way, when >90% of the materials of

Table 8. *Loss of V-factor activity with inactivation of cozymase*

(Smaller quantities of streptococci R were prepared as described in Table 5. In Exp. A, determinations were carried out on reaction solutions after centrifuging free from streptococci. In B, the separations indicated were carried out. Reactions were anaerobic.)

Reaction mixture (cozymase in 2·5 ml. with)	Cozymase determined with apozymase (mμmol./ml.)	V-factor activity (as mμmol. cozymase/ml.)
Exp. A		
(1) 0·015 M-Phosphates pH 6	17·3	17·8
(2) As (1), with streptococci (6·5 mg.); 40 min. at 37°	8·2	7·8
(3) 0·015 M-Phosphates, pH 7, with streptococci (6·5 mg.); 10 min. at 37°	13·9	12·2
(4) As (3), for 40 min.	1·7	1·7
Exp. B		
(1) 0·011 M-Phosphates, pH 7·6	11·0	12·0
(2) As (1), with streptococci (8·3 mg.); 40 min. at 38°, determination in solution	0·8	1·0
(3) Cells from reaction (2)	13·0	17·4
(4) Streptococci (8·3 mg.), before reaction	16·9	17·3

Table 9. *Nicotinamide from the streptococcal breakdown of cozymase*

(Exp. B is that of Table 5A, where further details are given. The cozymase of specimens which were taken to follow the course of the reaction has been subtracted from that added. A similar reaction mixture was employed in Exp. A, but with a different batch of organisms and with nicotinamide in place of cozymase. Nicotinic acid picrolonate depresses the melting point of that of nicotinamide.)

	Experiment A	Experiment B
Added ...	Nicotinamide, 52 μmol.	Cozymase, 31·5 μmol.
Isolated as fairly pure picrolonate of nicotinamide; m.p. 214–217°, not lowered by mixing with the pure substance (μmol.)	17	16
Isolated as less pure material, m.p. 208° or greater, not lowered by mixing with nicotinamide picrolonate (μmol.)	16	5
Picrolonic acid content of purer product, by titration with methylene blue (Bolliger, 1939; theory for nicotinamide 68·5%) (%)	69	70
Yield of nicotinic acid from purer picrolonate, determined by *Lactobacillus arabinosus* (theory for nicotinamide picrolonate, 31%) (%)	—	28

nicotinic acid activity were obtained in ethanolic solution. This solution was evaporated to *c.* 0·5 and 2·5 ml. of 1% (w/v) picrolonic acid in 50% aqueous ethanol added, and the solution kept cold. Yellow crystals separated; further material was obtained from the mother liquors by evaporation and addition of more picrolonic acid. By one recrystallization from aqueous ethanol the fractions of Table 9, Exp. B, were obtained. These indicate that the bulk of the product which has nicotinic acid activity is nicotinamide, and that its yield and purity are comparable to that of nicotinamide isolated from a similar reaction mixture to which it had been added as such, in place of cozymase (Exp. A). Exp. A was carried out first to serve as a model in the isolation of the product from cozymase. The data of Table 9 afford independent evidence for the stability of nicotinamide under conditions in which cozymase is inactivated.

splitting analagous to that in the liberation of nicotinamide from cozymase would lead to the formation of nicotinic acid itself, small quantities of which can be detected. Any reaction in trigonelline brought about by streptococci, however, aerobically or anaerobically, was of $<1/1000$ of the rate of the organisms' reaction with cozymase.

Inactivation of cozymase by Haemophilus parainfluenzae

The change brought about by this organism appears similar to that caused by streptococci. The course of loss of cozymase activity from solutions initially about 5×10^{-5} M was paralleled closely by loss of V-factor activity (Table 10). The nicotinic acid content of a similar reaction mixture did not fall

Table 10. *Breakdown of cozymase by* Haemophilus parainfluenzae

(Strain 4101 was employed and grown with the addition of 3% of yeast extract; initial concentration of cozymase in the medium, 2 μg./ml. The organisms were washed twice with, and suspended in, 0·9% NaCl. The quantity of cozymase indicated was added with phosphates (to 0·03 M) at pH 7. Incubation was aerobic at 37°. Determinations refer to the whole mixture, including bacteria. Cozymase itself remained unchanged in the absence of bacteria.)

Batch of organisms; time of growth; quantity used in reaction (dry wt.)	Time of reaction (min.)	Initial cozymase (mμmol./2·5 ml.)	% remaining of		
			Cozymase by apozymase	V-factor (as cozymase)	Nicotinic acid
A; 40 hr.; 4·2 mg.	0	120	(100)	(100)	—
	90	120	56	53	—
	180	120	19	19·5	—
	330	120	4	7	—
B; 46 hr.; 10·5 mg.	0	129	(100)	—	(100)
	90	129	14·4	—	97
	180	129	<3	—	100
	330	129	<3	—	104

Other changes during inactivation of cozymase; other substrates. We have found changes in the phosphate of streptococcal suspensions to be too great to obtain dependable information on the possible liberation of inorganic phosphate from cozymase during its inactivation by the organisms.

The possible production of acid during the reaction was examined by following the evolution of CO_2 from a medium containing 0·038 M-bicarbonate in equilibrium with 5% CO_2 in N_2. During the inactivation of 1·7 μmol. cozymase, 2·35 μmol. CO_2 were evolved from a vessel containing streptococci, and 2·43 μmol. from one also containing the cozymase. A similar reaction mixture containing yeast nucleic acid (3 mg./ml.), instead of cozymase, yielded CO_2 in large excess of a control without substrate, and corresponding to acid formation at the rate of 300 mμmol./mg./hr. Thymus nucleic acid also reacted; any reaction with yeast adenylic acid and guanylic acid was much slower. The yeast nucleic acid was purified according to Gulland & Jackson (1938), and acid formation from it was not inhibited by 0·08 M-azide, which reduced glycolysis to about 35% of its normal value. The streptococci thus appear to contain a nuclease, but their reaction with cozymase is of a different type from that with nucleic acids.

Pyridine N linked to carbon is found in other natural products. Of these, trigonelline was examined, since a

during a period at least twice as long as that which sufficed to inactivate almost all its cozymase. Breakdown therefore does not appear to lead to accumulation of nicotinamide riboside, but it does not extend beyond nicotinic acid.

Effect of nicotinamide on the streptococcal breakdown of cozymase: synthesis of cozymase

The effect of nicotinamide on the streptococcal breakdown of cozymase was examined since nicotinamide is a product of the reaction, and since it has been found to inhibit the analogous breakdown in animal tissues (Mann & Quastel, 1941; Handler & Klein, 1942). Retardation of the streptococcal reaction was found, but only with extremely high concentrations of nicotinamide. The retardation by 0·1 M-nicotinamide (2000 times the substrate concentration) was of the order of 50% (Table 11). Nicotinic acid salts had little or no action at 0·033 M.

Other products of cozymase breakdown by streptococci remain unknown, but we have attempted to obtain a synthesis of the material from nicotinamide and yeast adenylic acid. Table 11 shows that synthesis did not occur from these compounds alone,

Table 11. *Nicotinamide in the breakdown and synthesis of cozymase*

(Streptococci (strain R) were used on their first day of growth; reactions were aerobic and at 37°.)

Batch of organisms; conditions of reaction; time of growth	Reaction mixture: Substances added	Reaction mixture: Initial cozymase	Cozymase remaining (%)
A; pH 7·5; 60 min.	0	$4·8 \times 10^{-5}$ M	14
	Nicotinamide, 0·1 M	$4·8 \times 10^{-5}$ M	52
	Nicotinamide, 0·033 M	$4·8 \times 10^{-5}$ M	22
	Na nicotinate, 0·033 M	$4·8 \times 10^{-5}$ M	17
B; pH 7·5; 40 min.	0	5×10^{-4} M	26
	Nicotinamide, 0·055 M	5×10^{-4} M	47
	Nicotinamide, 0·028 M	5×10^{-4} M	29
			Change in cozymase (mμmol.)
C; pH 7·5; 40 min.	0	22 mμmol. (in cells)	—
	Adenylic acid, 0·0033 M; Nicotinic acid, 0·0033 M or both	22 mμmol. (in cells)	<1
C; pH 7·5; 10 min.	Adenylic acid, 0·0033 M; Nicotinic acid, 0·0033 M and glucose 0·033 M	22 mμmol. (in cells)	+12·4
C; pH 7·5; 40 min.	As above	22 mμmol. (in cells)	+26

but did take place when glucose also was added. In a similar reaction mixture the course of reaction was followed in more detail and the cozymase was found to increase at the rate of 20 mμmol./mg. dry wt./hr. during the first 50 min.

DISCUSSION

Streptococcal breakdown of cozymase. Inactivation of cozymase has been shown to be a very general reaction in bacteria. The reaction in streptococci reached surprisingly high velocities. With cozymase at 4×10^{-4} M, it was decomposed at rates about 400 mμmol./mg. of organisms/hr., equivalent to the decomposition during 1 hr. of a quantity of cozymase one-quarter of the bacterial dry weight. Studies of the essentials for microbial growth, and the development of microbial methods for estimating vitamin-like compounds, have tended to give the impression that bacteria normally react with only small quantities of such compounds. This is true only in one sense. Thus, the minimal concentration of nicotinic acid or its derivatives normally needed for maximal bacterial growth is less than 10^{-7} M. This value holds also for the streptococci studied here, though the quantity of cozymase in their cells was of the order of 5–10 mμmol./mg. dry wt. (Tables 2 and 6). The internal concentration of cozymase, assuming a water content of 80%, was therefore about $1–2 \times 10^{-3}$ M, or about 0·1% of the cell weight. These are not exceptional values; cozymase contents reported for yeast (cf. Sumner, Krishnan & Sisler, 1946) correspond to internal concentrations of 7×10^{-4} M. The coenzymes of red blood cells, normally of *c.* 10^{-4} M, can be raised to 3×10^{-4} M by feeding nicotinic acid (Handler & Kohn, 1943). The latter values are for substances of V-factor activity, presumably representing the sum of coenzymes I and II. Thus, although the rapid decomposition of cozymase by streptococci was observed with concentrations greater than 10^{-4} M, cozymase frequently occurs naturally at such concentrations.

It is therefore feasible that the enzymic activity concerned may play a part in the normal economy of the cells, even though substrate saturation is not attained at a cozymase concentration of 4×10^{-4} M. It is presumably of advantage to an organism to be able to interconvert the naturally occurring nicotinic acid derivatives. An alternative possibility which we considered, but now think unlikely, was that the decomposition of cozymase might occur by the action of a nucleotidase, and although the streptococci produced acid from nucleic acids, acid from cozymase was not formed at a comparable rate. It is certain also that nicotinamide is produced as such from cozymase, and that this involves breakage of the molecule between the pyridine N and the C_1 of ribose. This linkage is known to occur naturally only in the two coenzymes. The somewhat similar link between pyridine N and $—CH_3$ in trigonelline was split, if at all, at less than 1/1000 of the rate at which cozymase was inactivated. Liberation of nicotinamide from cozymase may be preceded or followed by changes in the remainder of the cozymase molecule. Our present experiments do not give much information on this subject, except that nicotinamide riboside did not accumulate during the breakdown of cozymase. Breakdown of cozymase by acids or alkalis also liberates nicotinamide as such (Schlenk, 1943).

Synthesis of cozymase by streptococci. Streptococci were able to synthesize cozymase in spite of the rapid breakdown of added cozymase. This is implied in the finding that cells grown in presence of nicotinic acid contained cozymase, as also did non-proliferating suspensions of cells, and it seems that the cozymase initially associated with streptococcal cells was not decomposed so rapidly as the added material. An enzyme inactivating cozymase was not, however, liberated to solution by the streptococci, nor easily extracted from them, and decomposition of cozymase may always be preceded by its assimilation by the cells (cf. Table 6). The observed rate of accumulation of cozymase under conditions permitting its synthesis is therefore likely to be less than the true rate of synthesis.

The maximum rate of accumulation of synthesized cozymase which was observed was 20 mμmol./mg. dry wt./hr. The streptococci used grow, when under satisfactory conditions, with generation times of 20–30 min. The rate of reaction (Q) in mμmol./mg./generation time is therefore 6·7–10. As indicated by McIlwain, Roper & Hughes (1948), this means that the cells produced by such growth could have a quantity of cellular cozymase of $q = Q \log_e 2$, or in this case of 4–6 mμmol./mg. dry wt. This is about the cell content actually observed (Table 2).

Breakdown of cozymase by other organisms. Findings with respect to the breakdown of cozymase in bacteria and in animal tissues are similar, though in neither are they very extensive. Brain suspensions, like the streptococci, liberated nicotinamide (Handler & Klein, 1942). *Haemophilus parainfluenzae* produced either nicotinic acid or nicotinamide. The reaction in animal tissues, as in bacteria, was rapid. The breakdown by rat brain, and probably by cobra venom, was inhibited by nicotinamide (Mann & Quastel, 1941), and we found a lesser breakdown by streptococci in the presence of nicotinamide, though high concentrations of this were required.

The bacteria which we found to inactivate cozymase represent many taxonomic groups: streptococci, staphylococci, *Escherichia*, *Proteus* and *Haemophilus*. They are also very varied in their reactions to nicotinic acid and its derivatives in growth: *Escherichia coli* not requiring such addition for growth, *Proteus morganii* and the cocci requiring nicotinic acid, and *Haemophilus parainfluenzae* requiring nicotinamide riboside. For this reason, breakdown of cozymase by *H. parainfluenzae* has a significance which is different from that of the breakdown by the other organisms. Reaction with cozymase by *H. parainfluenzae* was at about the same rate as by the other bacteria, but whereas these were capable of resynthesizing cozymase from the products of its breakdown, *H. parainfluenzae* could not do so. It thus inactivates rapidly a compound which is indispensable to its growth. This situation, which is presumably of ecological importance, has parallels in the behaviour of other organisms to other growth essentials (McIlwain, 1947).

One reason for our study of the breakdown of cozymase by *H. parainfluenzae* was as follows. Lwoff & Lwoff (1937) concluded from indirect evidence that added coenzymes I and II could be interconverted by this organism, though after assimilation such interconversion was not possible. Production from added cozymase of a simpler substance of V-factor activity would have provided a mechanism for the interconversion, but we were unable to demonstrate the production of such a substance.

SUMMARY

1. Cozymase at concentrations of about 10^{-4}–10^{-5} M was inactivated by streptococci, staphylococci, *Escherichia coli*, *Proteus morganii* and *Haemophilus parainfluenzae* (but not by *Lactobacillus arabinosus* 17–5) at rates of 5–30 mμmol./mg. dry wt. of cells/hr. at pH 6–7·5 and 37°.

2. Streptococcal cells have been found to be about 10^{-3} M with respect to cozymase, which was the form in which most of their nicotinic acid existed. Streptococci inactivated 4×10^{-4} M-cozymase at the rate of 400 mμmol./mg./hr., and so could inactivate their own dry weight of the substance in 4 hr.

3. Nicotinamide was produced from cozymase during its inactivation by streptococci. A nicotinamide derivative of V-factor activity, such as the riboside, did not accumulate. The breakdown by *Haemophilus parainfluenzae* was similar.

4. The breakdown in several bacteria was not greatly influenced by air (but was sometimes greater anaerobically), or glucose (this sometimes retarded it), and was still rapid at 10°.

5. Streptococci were able to synthesize cozymase from nicotinic acid and adenylic acid in the presence of glucose; rates of +20 mμmol./mg. dry wt./hr. were observed, which were about those required to account for the observed cell content of the substance.

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The Amylose Content of the Starch Present in the Growing Potato Tuber

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During the last few years it has been shown that starch consists of two components: amylose, which is a linear polymer and comparable with cellulose, and amylopectin, which is a branched chain polymer comparable with glycogen. The advances in this field have been greatly assisted by the development of the potentiometric iodine titration method for estimating the percentage of amylose in starch (Bates, French & Rundle, 1943; Hudson, Schoch & Wilson, 1943). By use of this procedure the amylose content of many starches has been determined, and starches have been examined in which the amylose content varies from less than 1% in the waxy starches to over 50% in certain varieties of pea starch (Bates *et al.* 1943; Hilbert & MacMasters, 1946). The published values of amylose contents for starches of similar botanical origin, e.g. maize, sometimes differ and this suggests that the amylose content is not necessarily a constant property of the starch from any given type of botanical species. Furthermore, by use of the newer methods for end-group determination, and with a knowledge of the amylose content of a starch, it is now possible without separation of the components to determine the proportion of glucose residues present in the amylopectin fraction of the starch as terminal groups (Brown, Halsall, Hirst & Jones, 1948). For example, the sample of rice starch which was examined by Hirst & Young (1939), who used the methylation technique, has now been shown to contain 12% of amylose, and hence we have present in the amylopectin fraction one non-reducing terminal group/26 glucose residues. In other samples of rice starch, however, an amylose content of 14–15% (Brown *et al.* 1948) has been found, and the amylopectin fraction has been shown to contain one non-reducing terminal residue/20 glucose residues. This indicates that different varieties of rice starch do not all contain the same type of amylopectin. It will be recalled that Meyer & Heinrich (1942) have claimed that the starches extracted respectively from potato tubers, leaves and shoots, contain different pro-

portions of amylose and amylopectin, the shoots in particular having a high proportion (approx. 46%) of the amylose component.

In view of this evidence it was considered desirable to investigate the amylose content and the ratio of terminal residues to non-terminal residues present in the starch of two different varieties of the same botanical species throughout their period of growth. We therefore decided to examine at frequent intervals during the growing season the starch present in the tubers of two varieties of potatoes, one an early and the other a main crop variety.

EXPERIMENTAL

Growing of potatoes. The potatoes were grown from virus-free Golden Wonder and King Edward 'seed', which was very kindly given to us by the Seale Hayne Agricultural College.

Isolation of the starch. The potatoes (*c.* 600 g.) were washed to remove soil, scraped to remove the skin and cut into small pieces, which were broken up in an ordinary mincing machine. The coarse mash thus obtained was filtered and then ground up with a little water in a mill. The resulting fine slush was filtered, and allowed to stand for some hours in 1% sodium sulphite solution with intermittent shaking. The solid matter was then allowed to settle out and the supernatant liquid, which was usually dark in colour, was decanted. This process was repeated two or three times until the sulphite solution no longer developed any colour. The solid matter was then suspended in water and filtered through coarse muslin, when fibrous material remained on the cloth whilst the starch granules passed through. They were allowed to settle and then washed by decantation with distilled water, the water being poured off before complete sedimentation had occurred. This procedure ensured that any of the less dense fibrous material which had come through the filter was removed from the starch. Finally, the starch was filtered off on a hardened smooth filter paper to avoid contaminating it with cellulose fibres, washed first with water and then with methanol, and dried under diminished pressure. The yield of starch was of the order of 40–60 g. More starch of a poorer quality (e.g. slightly discoloured) could be obtained by repeating the above extraction process with the fibrous material left on the muslin filter.

Investigation of the starches. All starch samples were first defatted by treatment in a Soxhlet apparatus for 18 hr. with a constant boiling mixture of dioxan (80%) and water (20%) (Schoch, 1942, 1946). The sulphated ash content of the fat-free starch was determined.

The amylose contents of the starch samples were determined by means of the iodine potentiometric method of Bates *et al.* (1943) as modified by Hudson *et al.* (1943). In our determinations a Cambridge potentiometer reading to 0·1 mV. was used in conjunction with a mirror galvanometer. In the calculation of the amylose content the amylose was assumed to take up 21·5 g. iodine/100 g. amylose (Higginbotham & Morrison, 1947). In many of the previous publications on this subject a lower figure (*c.* 19·2 g. iodine/100 g. amylose) has been used for the calculation of amylose content, and the percentages of amylose quoted will therefore be a little high. It is probable that some of the work which has been reported as having been carried out with pure amylose refers in fact to samples of amylose containing 5–10% amylopectin.

In addition to determining the amylose content certain other properties were investigated with certain of the starch samples. With these samples nitrogen estimations (micro-Dumas) were carried out to see whether any appreciable amount of protein was being isolated along with the starch. The optical rotations of these samples in N-NaOH were also determined, and finally the samples were oxidized by KIO_4, and from the amounts of formic acid produced the number of glucose residues containing one non-reducing terminal

Table 1. *Properties of starches from two varieties of potatoes*

Date of collecting potatoes	Ash content (%)	N %)	$[\alpha]_D^{20°}$ in N-NaOH	Amylose* (%)	A†	B†
			Golden Wonder potatoes			
10. vii. 46	0·8	< 0·1	+160°	18	32	26
16. vii. 46	1·2	—	—	18	—	—
24. vii. 46	0·8	—	—	19	—	—
31. vii. 46	0·9	—	—	19	—	—
7. viii. 46	1·2	< 0·1	+169°	18	33	27
14. viii. 46	0·5	—	—	16	—	—
20. viii. 46	0·4	—	—	16	—	—
5. ix. 46	0·7	—	—	16	—	—
10. ix. 46	0·9	< 0·1	+160°	16	32	27
			King Edward potatoes			
31. vii. 46	0·6	< 0·1	+159°	16	32	27
7. viii. 46	1·1	—	—	18	—	—
14. viii. 46	0·7	—	—	15	—	—
20. viii. 46	0·6	< 0·1	+159°	15	33	28
5. ix. 46	0·8	—	—	16	—	—
10. ix. 46	0·5	—	—	16	—	—
24. ix. 46	0·8	0·4	+161°	17	33	27

* The estimated error in the figures of amylose contents is ±1%, e.g. 18±1% amylose.

† Col. A gives the number of glucose residues/one non-reducing terminal residue in the whole starch, and col. B the corresponding figure for the amylopectin fraction. The estimated error in the end-group determinations is ±2 in 30 glucose residues, e.g. 32±2 glucose residues/one non-reducing terminal residue.

residue was calculated for the various samples. From these figures and the percentages of amylose present the number of glucose residues containing one non-reducing terminal residue in the amylopectin fraction was estimated. The results of the analyses and calculations are given in Table 1.

DISCUSSION

The figures for the amylose content of the starch for King Edward potatoes varied between 15 and 18 %, but showed no definite upward or downward trend. In the case of the starch for Golden Wonder potatoes the upper and lower figures were 19 and 16 %, respectively, and the lower values were observed at the later period of growth. The variation is, however, only just outside the estimated range of experimental error, and it is not possible at this stage to decide whether or not it is significant. For neither of these starches can it be said, on the basis of the present data, that any noticeable change in the proportion of amylose and amylopectin occurs during the period of growth. Furthermore, the results indicate that the amylopectins present in the two varieties of potato are identical, and belong to that group of amylopectins which contain some 24–26 glucose residues/terminal group. This proportion of end-groups is the same as that found also in other samples of potato starch (Brown *et al.* 1948).

SUMMARY

1. Samples of the starches present in the tubers of two varieties of potato, Golden Wonder and King Edward, have been isolated at intervals throughout the period of growth of the plants.

2. No significant variation in the properties of amylose (*c.* 17 %) and amylopectin (*c.* 83 %) was observed during the period of growth.

3. In the amylopectin portion of the starches there was one terminal glucose residue for every 24–26 glucose residues, and the amylopectin for the two varieties of potatoes appeared to be identical.

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The Sorption of DDT and its Analogues by Chitin

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On the basis of a general correlation between the possession of a chitinous cuticle and sensitivity to DDT poisoning, and the fact that chitin will sorb DDT from colloidal suspension, Richards & Cutkomp (1946) put forward the hypothesis that the chitin of the cuticle serves to concentrate DDT selectively from the media with which the insects are treated, and so to give a higher concentration inside the animal.* If this is actually the case then it is reasonable to expect that the toxicity of compounds similar to DDT will be affected in some degree by the extent to which they are sorbed by chitin, and that, in making comparisons of their potencies account must be taken of the degree of concentration of these substances in the insect by the sorptive action of the chitinous cuticle.

Experiments were therefore carried out to observe the sorption by chitin of a number of DDT analogues from colloidal suspensions. In order to do this it was necessary to devise a method for estimating the DDT or its analogues sorbed from colloidal suspension, and also to obtain a general picture of the effect of time, concentration and amount of chitin present on the amount of DDT sorbed.

* These authors say that DDT is adsorbed by chitin. This implies a surface action and since no proof is offered as to the mechanism of the process the term 'sorption' has been used throughout this paper since this word does not imply any particular mechanism.

Under the experimental conditions described here, it is possible that DDT is mechanically retained by chitin. The retention of DDT by chitin was therefore compared with that by fine sand, which would also retain DDT from colloidal suspension if a mechanical action only were involved.

In an attempt to obtain some information on the mode of sorption of DDT by chitin, the chitin was replaced by other materials, viz. cellulose, because of its similar structure to chitin, and wool representing a proteinous group of materials. Also, the sorption by chitin of different particle sizes was examined, but unfortunately it was not possible to measure the particle size of the chitin owing to the irregular size of the laminar particles.

The work described has been of a preliminary and exploratory nature upon which further investigations might be based, and it was not intended as an exhaustive examination of the problem of sorption of DDT and its analogues by chitin. Sufficient information was sought to decide whether or not the great difference in the toxicities of DDT analogues to insects might possibly be explained by differences in the rate or extent to which they are sorbed by chitin. Such differences were not observed, and experiments with further groups of analogues were not continued since it is unlikely that useful information would be obtained in this way. Further investigation on the effect of temperature on the sorption of DDT by chitin might, however, throw some light on the influence of temperature on the toxic action of DDT, and the work is to be continued with this end in view.

METHODS

Preparation of colloidal suspensions of DDT and its analogues. Colloidal suspensions of DDT may be prepared by the exchange of solvents method. Relatively stable suspensions, from which the disperse phase is not deposited for 24 hr. or more, may be prepared by rapidly diluting with water ethanolic solutions of DDT or its analogues. This can perhaps be best effected by running the solution from a funnel into a well stirred volume of water. Although the method allows of considerable control of the rate of dilution of the ethanolic solution, it presents difficulties when the amount of ethanol used is barely sufficient to dissolve at room temperature all the DDT or analogue present. In this case considerable quantities of solute are likely to crystallize out on the sides of the funnel, when the solvent evaporates from the large surface presented as the solution drains from the funnel. Moreover, in the circumstances under consideration, it is not possible to redissolve the deposited solute with more solvent. The use of hot ethanolic solution is also not practicable, since the greater evaporation from the hot solvent, combined with the rapid cooling by the glassware, tends to increase the amount of crystalline material separating out. It is, however, possible to prepare colloidal suspensions from ethanolic solutions of DDT analogues, saturated at room temperature, by adding water very rapidly to the vigorously stirred ethanolic solution. In this way separation of DDT analogue from the ethanolic solution before dilution with water is avoided.

In order to facilitate the rapid dilution of a small amount (5–10 ml.) of solution with a large bulk (100 ml.) of water, a special apparatus, made by cutting a 500 ml. round-bottomed flask through its maximum diameter, was constructed. The neck of the flask was then drawn out and sealed off to give a goblet-shaped vessel. To prepare a colloidal suspension, 10 ml. of ethanolic solution of DDT analogue were placed in the small cup formed by the sealed-off neck portion of the flask. The ethanolic solution was then stirred vigorously with a spiral glass stirrer driven by a small electric motor, and 90 ml. of distilled water were added as rapidly as possible from a measuring cylinder. An almost instantaneous dilution was obtained. A further 100 ml. of water were then added as rapidly as possible to give a colloidal solution of DDT analogue in 5% ethanol in water.

Sorption of DDT analogues on to chitin. The same general procedure was used throughout. A colloidal suspension of DDT, or one of its analogues under consideration, was freshly prepared and samples were measured out into conical flasks. Weighed portions of chitin (from lobster shell) were then added to the contents of some of these flasks, the remainder being used as blanks to correct for any analogue not in colloidal suspension. The flasks were then kept gently agitated on a mechanical shaking machine for the desired time.

For simplicity, the treatment of an individual flask and its contents will now be described, since from this point onwards the tests and blanks were treated in exactly the same way. After sorption had taken place for the desired period, the contents of the flask were filtered through a no. 3 sintered glass crucible, and any solids which remained in the flask were washed into the filter with distilled water. The washing of the filter with distilled water was continued until it was free from colloidal suspension. The DDT analogue retained by the filter was then estimated. Tests were also carried out with other materials instead of chitin.

Estimation of DDT or analogue sorbed by chitin. Of the methods available for the determination of DDT, only those based on the determination of either all or a definite part of the halogen of the molecule are directly applicable to DDT analogues. Other methods of estimating DDT are based on colour reactions, which may or may not be satisfactory for the estimation of its analogues. Therefore, since the removal of one molecule of HCl by alkali occurs very readily with both DDT and its analogues, the following method of estimating them was used in preference to any other method. The sintered glass crucible, through which the colloidal solution of DDT or one of its analogues had been filtered, was allowed to drain, and any water remaining in the underside was removed with a piece of filter paper. The crucible was then fitted into a dry holder held by a rubber bung in a Buchner flask, which contained a small test tube to collect the 5 ml. of acetone were used to dissolve any DDT or analogue retained by the crucible. The crucible holder was then washed with a little acetone, and the contents of the test tube were transferred to a 100 ml. stoppered conical flask, with further washing with acetone. The crucible was again washed with 5 ml. of acetone, which was collected in the test tube and transferred to the conical flask. The crucible was then washed successively with 0·5 N-ethanolic NaOH (1 ml.), ethanol (2 ml.) and acetone (5 ml.), and all washings collected in the test tube and finally transferred to the conical flask.

The solution in the conical flask was then evaporated to dryness on a water bath, not only to reduce the bulk of liquid, but also to ensure complete hydrolysis of the DDT or analogue present. In the meantime, the crucible had been washed with 5 ml. of distilled water, which were added to the residue in the conical flask. The liberated halogen was then estimated by the Volhard method. The contents of the conical flask were acidified with approx. 8 N-HNO_3 (0·5 ml.) and an excess of 0·01 N-$AgNO_3$ added. Nitrobenzene (1 ml.) was added, and the excess silver was titrated with standard 0·01 N-NH_4CNS using one or two drops of saturated ferric ammonium sulphate solution as indicator.

RESULTS

The results are presented in Tables 1–6.

Table 1. *The sorption of DDT by varying amounts of chitin*

(A colloidal suspension containing 0·05% DDT in 5% ethanol in water was prepared. Portions (20 ml.) of this suspension were agitated for 80 min. with various quantities of chitin, after which the amount of DDT sorbed by the chitin was estimated.)

Date	Chitin (g.)	DDT sorbed (corrected for blanks) (mg.)	DDT sorbed by chitin (mg./g.)	DDT in blanks with no chitin (mg.)
16. vii. 46	0·10	2·5	25	1·1
	0·20	2·6	13	0·6
	0·30	6·1	20	Mean 0·9
	0·40	7·6	19	
17. vii. 46	0·20	2·3	11	0·0
	0·30	4·1	14	1·0
	0·40	7·0	18	Mean 0·5
26. ix. 46	0·11	2·0	18	0·2
	0·21	3·3	16	0·6
	0·31	4·6	15	Mean 0·4
	0·39	6·6	17	
	0·50	8·0	16	—

Sand used instead of chitin (cf. 17. vii. 46)

Sand (g.)	DDT retained (not corrected for blanks) (mg.)
0·20	0·6
0·30	0·6
0·40	0·7

Table 2. *The sorption of DDT by chitin during various periods of time*

(Portions (0·3 g.) of chitin were added to 20 ml. portions of 0·05% DDT colloidal suspension in 5% ethanol in water. The mixtures were agitated for various lengths of time, after which the amount of DDT sorbed by chitin was determined.)

Date	Time of sorption (min.)	Chitin (g.)	DDT sorbed (corrected for blanks) (mg.)	DDT sorbed by chitin (mg./g.)	DDT in blanks with no chitin (mg.)
27. ix. 46	30	0·31	2·9	9	0·7
	60	0·31	4·5	15	0·2
	120	0·30	5·5	19	0·1
	240	0·31	6·5	21	1·4
					Mean 0·6
30. ix. 46	30	0·31	4·2	14	0·2
	60	0·31	5·6	18	0·1
	120	0·30	8·7	29	0·9
	180	0·30	9·0	30	0·1
	240	0·30	8·6	29	0·2
					Mean 0·5

Table 3. *The effect of varying the concentration of DDT on its sorption by chitin*

(Portions (20 ml.) of 0·5% DDT colloidal suspension in 5% ethanol in water were diluted with 5% ethanol to give various concentrations of DDT. To samples of these various dilutions, chosen to contain 10 mg. of DDT, portions of 0·3 g. of chitin were added and agitated for a period of 95 min.)

Date	DDT concentration (%)	Chitin (g.)	DDT sorbed (corrected for blanks) (mg.)	DDT sorbed by chitin (mg./g.)	DDT in blanks with no chitin (mg.)
2. x. 46	0·05	0·31	5·8	19	0·2
	0·025	0·31	2·3	7	0·7
					Mean 0·5
3. x. 46	0·05	0·32	6·7	21	0·4
	0·04	0·30	4·2	14	
	0·025	0·31	—	—	1·0
					Mean 0·7
4. x. 46	0·05	0·31	6·0	19	0·2
	0·04	0·31	4·6	15	
	0·033	0·30	3·4	11	
	0·025	0·30	1·6	5	
	0·013	0·31	1·8	3	1·1
					Mean 0·6

Table 4. *Sorption of DDT analogues by chitin*

(The sorption by chitin of the four symmetrically para-substituted halogen derivatives of 1:1:1-trichloro-2:2-diphenylethane from colloidal suspension in 5% ethanol in water was examined. Samples (40 ml.) of colloidal suspensions of the analogues, each containing $18{\cdot}6^* \times 10^{-6}$ mol. of analogue, were agitated with portions (0·30 g.) of chitin for various lengths of time, after which the amount of analogue sorbed by the chitin was estimated.)

Date	Time (min.)	Chitin (g.)	Analogue sorbed (corrected for blank) (mg.)	Analogue sorbed (corrected for blank) (mol. $\times 10^{-6}$)	Analogue in blank using no chitin (mg.)
	1:1:1-Trichloro-2:2-bis(4′-fluorophenyl)ethane; 0·0151% suspension				
23. x. 46	60	0·30	1·1	3·4	0·6
	115	0·30	1·1	3·4	
	150	0·29	2·0	6·2	
	195	0·30	2·4	7·5	0·7
	240	0·30	3·2	9·9	
	300	0·30	3·0	9·3	
	360	0·30	3·1	9·6	1·1
					Mean 0·8
	1:1:1-Trichloro-2:2-bis(4′-chlorophenyl)ethane; 0·0165% suspension				
18. x. 46	30	0·30	1·0	2·8	0·3
	60	0·30	1·4	3·9	
	90	0·29	1·6	4·5	
	150	0·30	2·0	5·6	
	180	0·30	2·3	6·5	
	210	0·30	2·9	8·2	
	240	0·30	2·5	7·0	
	270	0·30	2·2	6·2	0·1
					Mean 0·2
25. x. 46	60	0·30	0·9	2·5	0·5
	115	0·30	1·2	3·4	
	150	0·30	2·8	7·9	
	195	0·30	2·9	8·2	0·3
	240	0·30	3·0	8·5	
	360	0·30	4·2	11·8	0·0
					Mean 0·3

* $18{\cdot}6 \times 10^{-6}$ mol. of DDT or its analogue in 40 ml. of 5% ethanol in water was chosen so that these results would be comparable with the earlier experiments on the sorption of DDT and its analogues. It corresponds to 0·025% (w/v) suspension of the iodo analogue.

Table 4 (*cont.*).

Date	Time (min.)	Chitin (g.)	Analogue sorbed (corrected for blank) (mg.)	Analogue sorbed (corrected for blank) (mol. $\times 10^{-6}$)	Analogue in blank using no chitin (mg.)
	1:1:1-Trichloro-2:2-bis(4′-bromophenyl)ethane; 0·0206% suspension				
21. x. 46	60	0·30	1·7	3·8	1·0
	115	0·30	2·8	6·3	
	150	0·29	4·8	10·8	0·7
	195	0·30	3·8	8·6	
	240	0·30	4·4	9·9	
	300	0·29	5·2	11·7	
	360	0·29	5·6	12·6	0·0
					Mean 0·6
	1:1:1-Trichloro-2:2-bis(4′-iodophenyl)ethane; 0·0250% suspension				
11. x. 46	30	0·31	0·4	0·7	2·9
	60	0·30	1·5	2·8	1·2
	120	0·31	4·1	7·6	2·2
	180	0·30	6·4	11·9	0·0
	240	0·31	5·7	10·6	0·2
					Mean 1·3

(Amount of DDT sorbed in 4·5 hr. by 0·3 g. of chitin from a colloidal suspension containing $18·6 \times 10^{-6}$ mol. of analogue in 40 ml. of 5% ethanol in water.)

Amount sorbed (mol. $\times 10^{-6}$; corrected for blank)		Blank (mol. $\times 10^{-6}$)	
	1:1:1-Trichloro-2:2-bis(4′-fluorophenyl)ethane		
11·8		2·7	
10·9		4·6	
12·0		3·3	
11·7		4·1	
12·4			
Mean 11·8±0·6		3·7	
	1:1:1-Trichloro-2:2-bis(4′-chlorophenyl)ethane		
12·4		2·1	
11·3		2·3	
11·4		1·7	
9·4		2·4	
9·2			
Mean 10·7±1·4		2·1	
	1:1:1-Trichloro-2:2-bis(4′-bromophenyl)ethane		
11·5	13·0	1·7	0·2
11·6	14·2	2·3	0·0
15·7	13·9	1·1	
14·7			
Mean 13·4±2·2	13·7±0·6	1·9	0·1
	1:1:1-Trichloro-2:2-bis(4′-iodophenyl)ethane		
13·5		1·9	
14·2		1·5	
14·7		0·9	
15·5		2·0	
13·0			
Mean 14·2±1·0		1·6	
	1:1:1-Trichloro-2:2-diphenylethane		
10·9		3·2	
9·3		2·3	
12·1			
Mean 10·8		2·8	
	1:1:1-Trichloro-2:2-bis(4′-ethoxyphenyl)ethane		
7·3		0·8	
10·3		0·3	
8·4			
Mean 8·7		0·6	

Table 5. *Sorptive powers of chitin powder before and after grinding*

(A portion of chitin was taken from the original sample and ground in a mechanically operated agate mortar. Portions (0·1 g.) of the two samples of chitin were agitated with 20 ml. samples of 0·05% DDT suspension in 5% ethanol in water for 3 hr., and the amount of DDT sorbed by the chitin was estimated.)

DDT retained by chitin (mg./g., corrected for blanks)			
Before grinding		After grinding	
16	18	43	47
22	22	41	52
19	21	48	53
Mean 19±3	20±2	44±4	51±3

Table 6. *Action of various substances on colloidal suspension of DDT*

(Weighed portions of the substances under trial were agitated with 20 ml. portions of 0·05% DDT colloidal suspension in 5% ethanol in water. The amount of DDT retained was estimated immediately after addition of the material and at some later time. Blanks were also run to determine the amounts of DDT retained by the filters.)

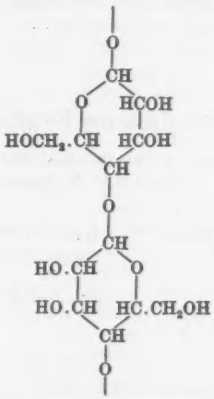
DDT retained (mg, corrected for blank)		
At zero time	After 2 hr.	Blank
Gooch asbestos, 0·10 g. portions		
6·0	1·8	0·8
1·1	−0·1	0·8
5·2	6·4	0·7
6·4	1·0	0·3
5·5	3·4	1·9
2·8	0·7	1·4
Sheep's wool, 0·20 g. portions		
−0·5	0·5	1·1
0·0	1·1	0·8
−0·4	1·4	—
0·0	0·9	0·9
0·0	1·2	0·8
0·0	0·6	0·8
Cellulose, (1) Fibres of cotton wool, 0·10 g. portions		
0·8	0·9	0·9
0·9	0·8	0·8
1·2	—	—
0·20 g. portions		
1·5	1·1	0·7
1·5	1·0	1·1
1·8	1·3	1·1
Cellulose, (2) Fibres from pulped filter paper, 0·10 g. portions		
7·8	7·7	0·6
8·3	8·8	0·5
8·3	8·6	0·6

	After 0·5 hr.	After 1·0 hr.	After 1·5 hr.	Blank
3·4	2·1	1·9	3·0	1·6
6·4	3·9	2·4	4·4	1·4

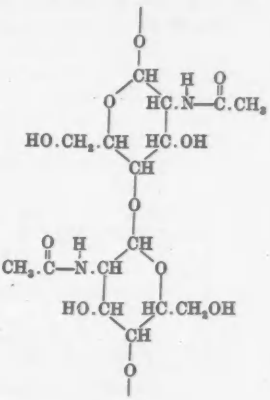
DISCUSSION

Since the agitation of sand with a colloidal suspension of DDT does not cause any significant increase in the amount of DDT retained by the filter itself from an untreated suspension (Table 1), it is clear that chitin must sorb DDT from its suspension, and does not cause the disperse phase to separate merely by mechanical action. The DDT retained by asbestos and filter paper fibres (Table 6) appears to be held mechanically, since the amount retained is apparently independent of time. If the action were other than mechanical, cellulose in other forms than filter paper pulp would be expected to retain DDT from colloidal suspensions, but cellulose in the form of cotton wool, in which the fibres are longer, and thus do not form such a compact pad, does not retain DDT. It therefore appears that DDT is not sorbed by cellulose. A small amount of DDT is retained by sheep's wool (Table 6), but much less than that sorbed by chitin. This rather weak affinity of wool for DDT is in line with the results reported by West & Campbell (1946).

Since the structural configuration of chitin and cellulose are very similar (Clark & Smith, 1936), it may be concluded that sorption occurs by a physicochemical or chemisorption process associated with the presence of amino groups in the sorbing substance.



Cellulose



Chitin

The relative lack of sorptive capacity of wool as compared with chitin may possibly be related to molecular configuration rather than to differences in surface area of equal weights of the two substances.

The increase in sorptive power of chitin as a result of grinding (Table 5) indicates that sorption is a surface phenomenon. Unfortunately, owing to the irregular shapes of the laminar particles, it was not possible to estimate the surface area of the chitin

powders, and thus to relate quantitatively surface area and the amount of DDT sorbed.

Comparison of the two sets of results in Tables 2 and 4 show that the rate at which the initial rapid sorption takes place depends on the concentration of the suspension used. This is further borne out by the results set out in Table 3, which records the amount of sorption by a fixed amount of chitin in a fixed time from colloidal suspensions of differing concentrations.

Consideration of the amount of DDT sorbed by a given mass of chitin (Tables 2 and 4) suggests that sorption takes place rapidly at first, and then either proceeds much more slowly or ceases altogether. Although the rate of sorption of DDT by chitin is partly dependent on the concentration of the suspension used, the apparent cessation of sorption (Tables 2 and 4) can be explained on the basis of the chitin becoming saturated with DDT, which again is the only possible explanation for the apparently constant amount of DDT sorbed by a unit weight of chitin in some experiments (Tables 1 and 2).

Although the molecular amounts of DDT analogues sorbed by a given weight of chitin from equimolecular suspensions (Table 4) are not significantly different, there appears to be a tendency for the less toxic analogues to be sorbed to a greater extent than the more toxic analogues. The rates at which the various analogues are sorbed appear to be approximately equal, the differences being well within experimental error.

SUMMARY

1. DDT and its analogues are readily sorbed by chitin from colloidal suspension. The sorptive power appears to be peculiar to chitin since cellulose, wool (protein fibre) and silica powder do not possess this power.

2. The rate of sorption depends on the concentration of the colloidal suspension.

3. The total amount sorbed by a given weight of chitin appears to depend on the surface area of the chitin.

4. The molecular amounts of DDT and its analogues sorbed from suspensions of equimolecular concentrations are approximately equal, as are apparently the rates of sorption.

5. There is no significant correlation between the sorption of DDT analogues on chitin and their toxicity.

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Studies on Biological Methylation

11. THE ACTION OF MOULDS ON INORGANIC AND ORGANIC COMPOUNDS OF ARSENIC

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Methylation by moulds: general and selective

The conversion of arsenites, selenites and tellurites to trimethylarsine, dimethyl selenide and dimethyl telluride in cultures of *Scopulariopsis brevicaulis* has been described by Challenger & Higginbottom (1935) and in Parts I–VII and IX of this series (for references see Challenger, 1945). The theoretical aspects of the subject are discussed in Part VIII (Challenger, 1942, 1945). This methylating action is also exerted on salts of alkyl- and dialkyl-arsonic acids, $R\text{AsO(OH)}_2$ and $R_2\text{AsO.OH}$ (Challenger, Higginbottom & Ellis, 1933; Challenger & Ellis, 1935; Challenger & Rawlings, 1936) giving $R\text{As(CH}_3)_2$ and $R_2\text{AsCH}_3$, and on salts of the alkylseleninic acids, $R\text{SeO}_2\text{H}$ (Bird & Challenger, 1942) giving $R\text{SeCH}_3$. Thom & Raper (1932) reported that several other moulds volatilized inorganic arsenic, doubtless as trimethylarsine, although they did not examine the product. Some of these organisms have been studied by Bird & Challenger (1939, 1942) with similar results. *Penicillium chrysogenum*, *P. notatum* and *P. notatum* sp. produce dimethyl telluride from potassium tellurite and dimethyl selenide from both sodium selenite and methaneseleninic acid. The three last named moulds are, however, incapable of producing trimethylarsine from arsenious acid under the usual conditions. This recalls the statement of

Segale (1903) who had obtained a mould (unspecified) which produced a volatile unidentified selenium compound, but was inert towards arsenic. No other workers have recorded any examples of this selective methylation. Further instances are now presented, and the interesting fact has emerged that *P. chrysogenum*, *P. notatum*, *P. notatum* sp. and two strains of *Aspergillus niger* exert a selective action on compounds of arsenic and readily methylate sodium methylarsonate, $CH_3AsO(ONa)_2$, and in most cases sodium cacodylate, $(CH_3)_2AsOONa$ to trimethylarsine, but are inert to arsenious acid. It could, however, be suggested that this conversion is not necessarily a methylation, but might involve a reduction followed by a disproportionation:

(*a*) $RAsO(OH)_2 \rightarrow RAs(OH)_2$;
(*b*) $3RAs(OH)_2 \rightarrow R_3As + 2As(OH)_3$.

Thus, Grischkwitsch-Trochimovski (1928) states that the oxide C_2H_5AsO which results from the action of alkali on ethyldichloroarsine $C_2H_5AsCl_2$, is converted to triethylarsine and sodium arsenite. This possibility is, however, excluded by the behaviour of sodium ethylarsonate $C_2H_5AsO(ONa)_2$ and sodium allylarsonate $CH_2{:}CHCH_2AsO(ONa)_2$ in bread cultures of *A. niger* and of *Penicillium chrysogenum* respectively, dimethylethylarsine and dimethylallylarsine being produced in a pure condition. If reduction and disproportionation only had occurred, the products would have been triethylarsine and triallylarsine respectively. Had methylation of any arsenious oxide, simultaneously produced (see equation (*b*)), taken place, trimethylarsine would have been formed in addition. Since only dimethylethylarsine and dimethylallylarsine are evolved from the cultures, the reaction with these three moulds is therefore a methylation, as in the case of the analogous experiments with *Scopulariopsis brevicaulis*.

Three of the moulds under discussion, *Penicillium chrysogenum*, *P. notatum* and *P. notatum* sp. exert no such selective action on selenium compounds, but convert sodium selenite and methane- ethane- and propane-1-seleninic acids to dimethyl-, methylethyl- and methyl-*n*-propyl selenides respectively (Bird & Challenger, 1942). The inability of the five organisms mentioned above to methylate inorganic arsenic must be associated with the earliest stages of the methylation process, which, according to any theory of biological methylation, involves the formation of methylarsonic acid $CH_3AsO(OH)_2$. The green Penicillia and *Aspergillus niger* readily convert this compound to trimethylarsine. The selective action may be connected with the electron-releasing effect of the alkyl groups, which would render electrons more readily available on the arsenic atom and the oxygen atom to which it is attached, thus facilitating both reduction and attachment of a methyl group.

Aromatic arsonic acids and Scopulariopsis brevicaulis

Phenylarsonic acid yields no volatile arsine in cultures of *S. brevicaulis* (Challenger & Rawlings, 1936, 1937). It seemed possible that 2:4-xylylarsonic acid containing two electron-releasing methyl groups might be more reactive, but in bread cultures of *S. brevicaulis* no xylyldimethylarsine or other volatile arsine was produced.

We have now found that phenyldimethylarsine is not evolved from cultures containing phenylarsenoxide (C_6H_5AsO) or phenylmethylarsonic acid ($C_6H_5As(CH_3)OOH$). An explanation based on the difficult volatility of phenylmethylarsines would appear to be excluded, because it has now been shown that the nitrate of phenyldimethylarsine oxide ($C_6H_5As(CH_3)_2O$) is readily reduced by bread cultures of *S. brevicaulis* giving phenyldimethylarsine ($C_6H_5As(CH_3)_2$), which can be removed in sterile air and characterized as the mercurichloride. Were the mould able to methylate phenylarsonic acid to phenyldimethylarsine the oxide $C_6H_5As(CH_3)_2O$ would be an intermediate compound, on the basis of either the transmethylation or the formaldehyde theory of biological methylation (Challenger, 1942, 1945).

This easy reduction of phenyldimethylarsine oxide nitrate (phenyldimethylhydroxyarsonium nitrate) recalls the formation of trimethyl- and tri-*n*-propylarsines from trimethylhydroxyarsonium nitrate and tri-*n*-propylarsine oxide respectively (Challenger & Higginbottom, 1935), of diethyl sulphide from diethyl sulphoxide, and of dimethyl selenide from dimethylhydroxyselenonium nitrate (Challenger & North, 1934), in cultures of *S. brevicaulis*. On the other hand, trimethylamine oxide hydrate and trimethylhydroxyammonium chloride give no trimethylamine in bread cultures of this mould. In view of the enzymic formation of trimethylamine from its oxide in fish this negative result is interesting.

Attempt to test the formaldehyde hypothesis of biological methylation

This hypothesis assumes the formation of hydroxymethylarsonic acid ($HOCH_2AsO(OH)_2$) from arsenious oxide and its subsequent reduction to $CH_3AsO(OH)_2$. This acid has not been synthesized and a crucial test of the hypothesis has not been possible (Challenger *et al.* 1933; Challenger & Higginbottom, 1935). It appeared possible that the behaviour of the condensation product of phenylarsine ($C_6H_5AsH_2$) and acetaldehyde ($C_6H_5As(CHOHCH_3)_2$) (Palmer & Adams, 1922) in cultures of *S. brevicaulis* might throw some light on the problem, since this should yield phenyldiethylarsine. In glucose-Czapek-Dox cultures of the mould, however, the compound underwent hydrolysis yielding acetalde-

hyde, its instability recalling that of the condensation product of ethylthiol and acetaldehyde (Levi, 1932; Challenger & Rawlings, 1937). The behaviour of this hydroxyarsine to mercuric chloride was studied in view of its possible volatilization from the cultures and reaction with this reagent in the absorption train. With excess of a saturated aqueous solution the arsine gave a white precipitate, which, on boiling with ethanol, gave mercury, mercurous chloride, phenylmercury chloride, arsenobenzene, acetaldehyde, and a trace of a phenylhalogenarsine, probably phenyldichloroarsine.

It is well known that choline and betaine can engage in transmethylation in rats (Simmonds, Cohn, Chandler & du Vigneaud, 1943; du Vigneaud, Simmonds, Chandler & Cohn, 1946), and mycological methylation may proceed by a similar process (Challenger, 1945). No appreciable effect on trimethylarsine production was observed by Challenger & Higginbottom (1935) on addition of choline or betaine to bread cultures of *S. brevicaulis* containing arsenious oxide. The effect of 'triethylcholine' chloride $[(C_2H_5)_3NCH_2CH_2OH]^+Cl^-$ and 'triethylbetaine' $^+(C_2H_5)_3NCH_2COO^-$ in similar cultures has now been investigated. Careful examination of the dimercurichloride so obtained failed to show the presence of an ethyl derivative. This recalls the work of du Vigneaud (1941), who showed that when ethionine (*S*-ethylhomocysteine) and choline are fed to rats on a methionine-free diet no growth resulted, indicating that de-ethylation of ethionine to homocysteine does not occur. Results of the present and earlier papers are summarized in Table 1.

EXPERIMENTAL

Behaviour of different moulds in bread cultures to various sources of arsenic

Unless otherwise stated the moulds were obtained from the National Collection of Type Cultures at the Lister Institute, London (N.C.T.C.).

Arsenious oxide. The following moulds were found to convert As_2O_3 to trimethylarsine when the oxide was present to the extent of about 0·2% in the bread: *Aspergillus glaucus* (Birkbeck) (N.C.T.C. no. 1216) and *A. versicolor* (N.C.T.C. no. 1883). The quantity produced was, however, much smaller than with the organisms and substrates described below. The trimethylarsine was absorbed in acid $HgCl_2$ (Biginelli's solution) (Challenger *et al.* 1933), and characterized as the dimercurichloride; the melting point (decomp.) of the unrecrystallized product varied from 255 to 260°. Satisfactory mixed melting points were carried out in these and most of the following experiments. Trimethylarsine dimercurichloride has been repeatedly examined during the course of these investigations (Challenger *et al.* 1933; Challenger & Higginbottom, 1935). We find that, using bread cultures of *Scopulariopsis brevicaulis* (Sacc.) Bainier (*Baarn List of Fungi*, 1932) containing about 0·1% of As_2O_3, roughly 70% of the added arsenic may be recovered as trimethylarsine dimercurichloride during

Table 1. *Behaviour of different moulds to various sources of arsenic, selenium and tellurium in bread cultures*

Mould	N.C.T.C. no.	Compound added to culture: As_2O_3	Na_2HAsO_4	Na_2SeO_3	K_2TeO_3	$CH_3AsO(ONa)_2$	$(CH_3)_2AsO(ONa)$	$C_2H_5AsO(ONa)_2$	$C_3H_5AsO(ONa)_2$
		Identified product							
S. brevicaulis	1362,580	$(CH_3)_3As$	$(CH_3)_3As$	$(CH_3)_2Se$	$(CH_3)_2Te$	$(CH_3)_2As$	$(CH_3)_2As$	$C_2H_5As(CH_3)_2$	$C_2H_5As(CH_3)_2$
P. notatum	4222	None	—	$(CH_3)_2Se$	Doubtful $(CH_3)_2Te$, odour only	$(CH_3)_3As$	$(CH_3)_3As$	—	—
P. chrysogenum	589	None	—	$(CH_3)_2Se$	$(CH_3)_2Te$	$(CH_3)_2As$	$(CH_3)_2As$	—	$C_2H_5As(CH_3)_2$
P. notatum sp.	—	None	—	$(CH_3)_2Se$	$(CH_3)_2Te$	$(CH_3)_3As$	Odour of $(CH_3)_2As$, only trace of $(CH_3)_3As$, $2HgCl_2$	—	—
A. niger	—	None	—	Na_2SeO_4	—	$(CH_3)_3As$	$(CH_3)_3As$	$C_2H_5As(CH_3)_2$	—
A. fischeri	3779	None	—	(No odour, no redn.)	(No odour, no redn.)	$(CH_3)_3As$	—	—	—
A. niger 17	—	—	—	—	—	$(CH_3)_3As$	$(CH_3)_3As$	—	—
A. glaucus (Birkbeck)	1216	$(CH_3)_3As$	—	(No odour, no redn.)	(No odour, no redn.)	$(CH_3)_3As$	—	—	—
A. versicolor	1883	$(CH_3)_3As$	—	(No odour, no redn.)	(No odour)	$(CH_3)_3As$	—	—	—

24 months' aspiration. *S. brevicaulis* (strain Derx N.C.T.C. no. 1362) yielded about 80% in 10 months. An attempt was made by Dr C. Simons in these laboratories to increase the rate of trimethylarsine formation by this mould by cultivating the strain *S. brevicaulis* (Sacc.) Bainier (*Baarn List of Fungi*, 1932) for 33 generations on a potato-agar medium containing 0·12–0·15% of As_2O_3. No appreciable alteration in the rate of methylation could be detected, when the 'trained' mould was inoculated on to sterile bread containing 0·1% of As_2O_3.

Sodium methylarsonate ($CH_3AsO(ONa)_2$). The following moulds gave trimethylarsine, characterized as the dimercurichloride (unrecryst.), the melting point (decomp.) varying from 252 to 263°: *Penicillium chrysogenum* (N.C.T.C. no. 589), *P. notatum* (N.C.T.C. no. 4222), *P. notatum* sp. (see Bird & Challenger, 1939), *Aspergillus niger* and '*A. niger* 17' (obtained from Dr T. K. Walker of the Biochemistry Department, The College of Technology, Manchester), *A. fischeri* (N.C.T.C. no. 3779), *A. glaucus* (Birkbeck) (N.C.T.C. no. 1216) and *A. versicolor* (N.C.T.C. no. 1883). The concentration of the sodium methylarsonate in the bread was 0·2–0·25% except in the case of *A. niger*, where it was 0·07%. In the case of *Penicillium notatum* and *Aspergillus niger* 17 the trimethylarsine was also characterized as trimethylhydroxyarsonium nitrate $(CH_3)_3As(OH)NO_3$ (Challenger *et al.* 1933), m.p. and mixed m.p. 124 and 126° respectively.

Sodium cacodylate ($(CH_3)_2AsOONa$). Trimethylarsine was again obtained and characterized as before in cultures of the following: *Penicillium chrysogenum*, *P. notatum*, *Aspergillus niger* and '*A. niger* 17'. The usual concentration was 0·2 and 0·25% in the bread. *Penicillium notatum* sp. gave only a trace of the dimercurichloride.

Ethylarsonic acid ($C_2H_5AsO(OH)_2$) at a concentration of 0·13% in the bread gave with *Aspergillus niger* dimethylethylarsine, characterized as the mercurichloride, m.p. and mixed m.p. 238° (Challenger *et al.* 1933).

Sodium allylarsonate ($CH_2{:}CHCH_2AsO(ONa)_2$), concentration 0·2%, gave with *Penicillium chrysogenum* dimethylallylarsine, characterized as the mercurichloride, m.p. and mixed m.p. 209°. Here again no reduction of the double linkage in the allyl group occurs (Challenger & Ellis, 1935).

Ethyl-n-propylarsonic acid ($C_2H_7(C_2H_5).AsOOH$), concentration 0·17% in the bread cultures, gave with '*Aspergillus niger* 17' methylethyl-*n*-propylarsine, characterized as the dimercurichloride, m.p. and mixed m.p. 171–172°, decomp. between 189 and 192° (Challenger & Rawlings, 1936).

n-Propylarsonic acid ($C_3H_7AsO(OH)_2$), concentration 0·34% in the bread cultures, gave with *Penicillium notatum* dimethyl-*n*-propylarsine dimercurichloride, m.p. and mixed m.p. 195–197°, decomp. 199–200° (Challenger & Ellis, 1935).

The non-occurrence of methylation of arsenious oxide with certain moulds

In preliminary experiments the following organisms appeared unable to convert As_2O_3 to trimethylarsine in bread cultures: *P. chrysogenum*, *P. notatum*, *P. notatum* sp., *Aspergillus niger*, '*A. niger* 17' (As_2O_3 = 0·02%) and *A. fischeri* (As_2O_3 = 0·2%). Nevertheless, it was decided to treat five of these organisms with varying concentrations of As_2O_3. Six cultures of each mould on 50 g. of bread without added water were incubated for 9 days, and a sterile aqueous solution of As_2O_3 (15 ml.; 20 ml. in F) added to each group of five flasks (groups A–F) to give concentrations in the bread of 0·02, 0·05, 0·10, 0·20, 0·30 and 0·40%. During the first 3–4 days the *Penicillium notatum* flasks D, E and F had a very faint odour, barely detectable on the 10th day. On the 14th day an odour was detected in flask C, though with difficulty. With *P. chrysogenum* a very faint odour appeared to be present in E and F. The two strains of *Aspergillus niger* gave no odour, nor did *Penicillium notatum* sp., until after about 2 months, by which time slight contamination may have occurred.

Appearance of the cultures. With the six cultures of *P. notatum* the bread was covered with mycelium about 3 days after addition of As_2O_3. After 9 weeks the cultures showed almost black patches, especially flasks D, E and F. Subcultures from these on to wort agar failed to grow during 45 days, although they were reinoculated on the 5th day. All the cultures of *P. chrysogenum* and *P. notatum* sp. grew well. The strains of *Aspergillus niger* grew well in groups A and B, but in D, E and F very little growth occurred after addition of the As_2O_3.

Penicillium notatum *and arsenious acid.* It was decided to test for trimethylarsine evolved by *P. notatum* by chemical means. Five 1 l. flasks, each containing 150 g. of bread, were sterilized, inoculated with spores of *P. notatum* and incubated for 7 days at room temperature. To each were added 45 ml. of sterile 1% (w/v) As_2O_3 solution (conc. in bread 0·3%), and aspiration commenced through two tubes of Biginelli's solution. No deposit appeared and no odour was detected during 30 days. The cultures in two flasks were then quite white, the green sporing area being completely covered. Subcultures were made from these two flasks, each to two wort-agar slopes, to see if this white growth was contamination or sterile mycelium due to the As_2O_3. After 5 days' incubation at room temperature all four slopes showed only typical green colonies of *P. notatum*. One plate culture was also made from each flask with the same result. *P. notatum* would therefore appear not to produce trimethylarsine from As_2O_3, in agreement with the original observation.

Growth of P. notatum *on a liquid medium containing arsenious oxide from the outset.* Seven 250 ml. conical flasks, each containing 100 ml. of 5% (w/v) glucose-Czapek-Dox solution with concentrations of As_2O_3 of 0·01, 0·02, 0·04, 0·08, 0·16, 0·32 and 0·64%, were sterilized, inoculated and incubated at room temperature. After 10 days all the flasks except the last showed a white surface growth which was compact, raised and abnormal. During another 4 days, growth continued in concentrations 0·01–0·16%; increase was slow with 0·32%.

Arsonic acids containing the phenyl group and Scopulariopsis brevicaulis

The strain used was designated *S. brevicaulis* (Sacc.) Bainier in the *Baarn List of Fungi* (1932), and was distinguished in the Leeds laboratories as strain A (Challenger *et al.* 1933). It was grown on 150 g. of bread in 1 l. flasks. Solutions containing the arsonic acids were made up thus: (*a*) Phenylarsonic acid (1·2 g.) carefully purified by three crystallizations from water, m.p. 161–162°, was dissolved in water (100 ml.) containing $NaHCO_3$ (1·1 g.) and sterilized. (*b*) 2:4-Xylylarsonic acid (0·6 g.) (Michaelis, 1902) was dissolved in sterile water (80 ml.) containing $NaHCO_3$ (0·65 g.). (*c*) Benzylarsonic acid (0·75 g.), three times recrystallized from water without change in m.p. 196–198° after air-drying (the acid readily loses water), was suspended in sterile water (80 ml.).

25 ml. of solutions (*a*) and (*b*) and of suspension (*c*) were added to bread cultures of the mould (three in each case), and volatile products aspirated through acid $HgCl_2$ solution. A precipitate formed after some weeks in each case, but was shown to be free from the mercurichloride of a tertiary arsine $RAs(CH_3)_2$ and to consist of the ammonia compound of $HgCl_2$ which was prepared for comparison.

Behaviour of arsenious oxide in bread cultures of Scopulariopsis brevicaulis *containing 'triethylcholine' chloride and the triethylbetaine of aminoacetic acid*

'Triethylcholine' chloride (25 ml. of a sterilized 8% (w/v) aqueous solution) was added to each of three flasks of sterile bread, which were then inoculated with the mould and incubated for 4 days at 32° and 4 days at room temperature. Each flask then received 25 ml. of a sterile aqueous 1·2% (w/v) solution of As_2O_3. Aspiration through Biginelli's solution gave during 46 days only 0·22 g. of trimethylarsine dimercurichloride, m.p. 266–267° decomp. A mixed melting point confirmed its identity. 'Triethylbetaine' (25 ml. of a sterilized 3% (w/v) aqueous solution) was added to each of four 1 l. flasks containing 150 g. of sterile bread, which were then inoculated and incubated for 4 days at 32° and 2 days at room temperature. Addition of 25 ml. of sterilized aqueous 1·2% As_2O_3 solution gave as before during 28 days 0·2 g. trimethylarsine dimercurichloride, m.p. 266–267° with sintering from 263°. The mixed melting point with an authentic specimen of m.p. 267° was 266°. On the 40th day a further deposit was removed, m.p. 265 and 266° after recrystallization from very dilute $HgCl_2$ solution. Similar results were obtained with two cultures containing 2% and one with 1% of added 'triethylbetaine' connected in series.

Scopulariopsis brevicaulis *and aromatic compounds of arsenic*

S. brevicaulis *and phenylarsonic acid*. The acid (4 g.) and Na_2CO_3 (2 g.) were dissolved in water (150 ml.). 25 ml. of the solution were added to each of six 1 l. flasks containing 3% (w/v) glucose-Czapek-Dox medium (250 ml.) on which a good mycelium had formed. Sterile air was then drawn through the flasks in series with Biginelli's solution during 30 days, but no deposit formed, indicating the absence of phenyldimethylarsine. The whole of the medium was then filtered, concentrated to 400 ml. and a portion tested for phenyldimethylarsine oxide with picric acid, but no precipitate formed. The main bulk was then extracted ten times with ether, which only removed traces of phenylarsonic acid. A portion of the concentrated medium gave with HCl, SO_2 and I_2 (see p. 82) a yellow oil, which with ethanolic $HgCl_2$ yielded phenylmercury chloride, m.p. and mixed m.p. 247° in admixture with a specimen obtained in a similar manner from authentic phenylarsonic acid (see p. 83). From the bulk of the concentrated medium 30% of the total phenylarsonic acid was recovered as the thorium salt (Rice, Fogg & James, 1926). Quantitative recovery was not expected due to the material having been used for preliminary tests. Removal of thorium as oxalate, and of oxalic acid with H_2O_2 yielded phenylarsonic acid. It sintered at 158°, in agreement with an authentic specimen.

S. brevicaulis *and phenylarsenoxide*. Addition of 0·2% of the oxide to each of four cultures of the mould prepared as in Exp. 1 gave no odour during 6 months, and the insoluble oxide appeared unchanged. Neither reduction to phenylarsine nor production of phenyldimethylarsine occurred.

S. brevicaulis *and phenylmethylarsonic acid*. For the preparation of the acid see Burrows & Turner (1920, 1921); Gibson & Johnson (1928). Five 1 l. conical flasks, each containing a good growth of the mould on 5% (w/v) glucose-Czapek-Dox solution (200 ml.), were treated with 25 ml. of sterile 2% aqueous sodium phenylmethylarsonate. There was no odour and no precipitate in aqueous $HgCl_2$ after aspiration for 5 weeks. Evaporation of the slightly acidified medium, extraction with ethanol and treatment with HCl, SO_2 and excess I_2 gave a yellow oil. This with ethanolic $HgCl_2$ yielded a white precipitate, which on recrystallization from ethanol sintered at 148°, but did not melt. Its behaviour was identical with that of the mercurichloride of authentic phenylmethyliodoarsine. (Found, for the specimen from the culture: C, 14·5; H, 1·8; halogen, 33·2. $C_6H_5(CH_3)AsI.HgCl_2$ requires C, 14·9; H, 1·42; halogen 35·0%.) The medium therefore appeared to contain unchanged phenylmethylarsonic acid or phenylmethylarsenoxide.

S. brevicaulis *and phenyldimethylhydroxyarsonium nitrate*. Six 1 l. flasks, each containing 150–200 g. of bread slightly moistened with water, were inoculated with an aqueous spore suspension of the mould (strain Saccharomyces Bainier, see p. 80). After 4 days the odourless solution of the hydroxyarsonium nitrate in sterile water was added, the concentration in the bread being 0·17%. In a few hours an arsenical odour was apparent, and the flasks were connected with sterile bungs and delivery tubes as in earlier experiments (Challenger *et al.* 1933). A stream of sterile air was then aspirated through the cultures into saturated aqueous $HgCl_2$. A deposit formed after 2 days, which when recrystallized from hot water had m.p. and mixed m.p. 196° with authentic phenyldimethylarsine mercurichloride, m.p. 196°. A second crop sintered from 185 to 195°. After one recrystallization the melting point rose to 196°. Later on, due to the production of NH_3 in the cultures, the precipitate increased rapidly. It was extracted with ethanol and then with boiling water leaving the ammonia compound of $HgCl_2$. The phenyldimethylarsine mercurichloride obtained (0·62 g.) represented 23% of the original hydroxyarsonium nitrate. In a control experiment six test tubes, each containing approximately 7 g. of sterile bread and 0·17% of the hydroxyarsonium nitrate, gave no odour or any deposit in aqueous $HgCl_2$ during 28 days.

S. brevicaulis *and phenylbis(1-hydroxyethyl)arsine*. The arsine (3 g.) was suspended in sterile water (150 ml.), and 25 ml. added to each of six well-grown cultures on glucose-Czapek-Dox solution (200 ml.). Sterile air was drawn over the cultures into saturated $HgCl_2$. After a few days the only odour recognizable was that of the original arsine. A slight precipitate in the absorption bottles was shown to be $HgCl_2$. On placing aqueous 2:4-dinitrophenylhydrazine hydrochloride at the end of the absorption train a yellow solid formed. When recrystallized from ethanol this melted at 156° alone and in admixture with authentic acetaldehyde 2:4-dinitrophenylhydrazone of m.p. 156°.

Reactions of some aromatic compounds of arsenic

Conversion of phenylarsonic acid to phenylmercury chloride. The acid was converted to phenyldiiodoarsine. In acetone this gave a white addition product with aqueous $HgCl_2$. Heating this with water gave a red solid and hot

acetone produced a red solution, doubtless due to HgI_2. Crystallization from ethanol yielded white glistening plates, m.p. 242°. On successive recrystallizations from acetone, ethanol and acetone, the m.p. were 247, 246 and 247°, not depressing the m.p. (251°) of phenylmercury chloride.

Phenyldiethylarsine mercurichloride. The arsine (*c.* 1 g.) (Burrows & Turner, 1920) was shaken with excess of saturated aqueous $HgCl_2$ until the mixture was odourless. The white precipitate was recrystallized from ethanol (m.p. 127, 129, 129°). (Found: Cl, 15·2; Hg, 42·2. $C_{10}H_{15}Cl_2AsHg$ requires Cl, 14·74; Hg, 41·65%.)

Phenylbis(1-hydroxyethyl)arsine and mercurichloride. A concentrated ethanolic solution of the arsine (Palmer & Adams, 1922) was shaken with excess of saturated aqueous $HgCl_2$ for several hours. The white precipitate quickly became yellow. It still smelled slightly of the arsine and was washed with water and a little ethanol. It blackened and decomposed from 90°. Warming with ethanol gave Hg_2Cl_2 and mercury but no Hg_2O. The ethanolic filtrate (B) gave white crystals, m.p. 254°; an identical product also slowly separated from the aqueous filtrate (A) from the crude mercurichloride. It contained halogen and mercury but no arsenic, and was shown by mixed m.p. (255°) to be phenylmercury chloride (m.p. 252°). Concentration of the filtrate (B) gave a white crystalline material, m.p. 187–194°, which was twice recrystallized from benzene-ether, m.p. 212 and 213°. It contained arsenic but no halogen or mercury, and was unaffected by NaOH, NH_3 or HCl. It was readily attacked by HNO_3, $KMnO_4$, H_2O_2 and I_2 and was insoluble in water. (Found: C, 48·3; H, 3·5; mol.wt. (camphor), 491. Calc. for $C_{12}H_{10}As_2$: C, 47·4; H, 3·3%; mol.wt. 304.) Arsenobenzene, m.p. 212°, gives high values in ebullioscopic molecular weight determinations in benzene (Michaelis & Schäfer, 1913). Its identity was confirmed (1) by oxidation with HNO_3 when the product (phenylarsonic acid) gave a white gelatinous thorium salt (see p. 82); and (2) by treatment with Cl_2 giving a yellow oil ($C_6H_5AsCl_2$) which soon crystallized ($C_6H_5AsCl_4$). A portion of the filtrate (B) with 2:4-dinitrophenylhydrazine hydrochloride gave a copious yellow precipitate, m.p. and mixed m.p. 159°, with authentic acetaldehyde 2:4-dinitrophenylhydrazone, m.p. 159°. This was also obtained from the aqueous filtrate (A). Complete evaporation of (B) left only a few drops of a yellow lachrymatory oil containing chlorine. It was probably phenyldichloroarsine.

SUMMARY

1. *Scopulariopsis brevicaulis* converts arsenious acid and methylarsonic acid to trimethylarsine; other alkylarsonic acids yield alkyldimethylarsines. *Penicillium notatum*, *P. notatum* sp., *P. chrysogenum* and two strains of *Aspergillus niger* are inert to arsenious acid, but can methylate alkylarsonic acids. *A. glaucus* gives trimethylarsine with arsenious and methylarsonic acids. All cultures were on bread.

2. Phenylarsonic and phenylmethylarsonic acids in glucose-Czapek-Dox medium, and *m*-xylylarsonic and benzylarsonic acids in bread cultures give no volatile arsenic compounds with *Scopulariopsis brevicaulis*. Phenyldimethylhydroxyarsonium nitrate, however, is readily reduced in bread cultures to phenyldimethylarsine which readily volatilizes.

3. In cultures of *S. brevicaulis* on glucose-Czapek-Dox medium phenylbis(1-hydroxyethyl)arsine does not give phenyldiethylarsine, but is hydrolyzed to acetaldehyde. The behaviour of the hydroxy arsine to mercuric chloride has been studied.

4. In bread cultures of *S. brevicaulis* containing arsenious acid and 'triethylcholine' chloride or 'triethylbetaine' only trimethylarsine is evolved; transethylation apparently does not occur.

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The Chemical and Immunological Properties of Phosphorylated Proteins

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The natural phosphoproteins such as casein and ovovitellin offer much of interest, chemically and immunologically, and many investigators have studied both these and artificially phosphorylated proteins, to determine how the phosphate groups are linked to the protein molecule; comparisons of the chemical properties of both types of protein have also been made by several workers. Phosphoryl chloride was used as phosphorylating agent by Bechhold (1901), Neuberg & Pollak (1910) and Neuberg & Oertel (1914). These methods were modified by Rimington (1927), who treated the ice-cold protein solution with phosphoryl chloride in carbon tetrachloride, adding alkali at intervals to keep the solution faintly alkaline. Rimington described the preparation and properties of phosphorylated proteins prepared from casein, denatured serum globulin and dephosphorized casein.

A comprehensive chemical and immunological investigation of phosphorylated egg albumin was made by Heidelberger, Davis & Treffers (1941). They found that phosphorylation, which was accompanied by some denaturation, effected a profound change in the serological reactions of the egg albumin. Immunological investigations have also been made on phosphorylated casein (Ecker & Simon, 1928) and casein, dephosphorized casein and rephosphorized casein (Kesztyus & Kocsis, 1942).

Our own investigations with phosphorylated proteins were started with two main objects. It was hoped that the introduction into the protein molecule of phosphate groups containing radioactive P would yield labelled antigens which could be used for serological investigations, e.g. for studies on the fate of injected protein antigens and for quantitative studies on the precipitin reaction. It was also intended to study the nature of the linkage between the phosphate group and the protein in phosphoproteins and phosphorylated proteins, by immunochemical and isotopic tracer methods. Some of the results of these investigations are given in the present paper.

EXPERIMENTAL

Phosphorylation and dialysis. The method used was essentially that described by Rimington (1927). Freshly distilled $POCl_3$, dissolved in about 8 vol. of carbon tetrachloride, was added slowly to the protein solution cooled in ice water; varying amounts of $POCl_3$ were used, usually ranging from 0·1 to 0·5 g./g. of protein. The mixture was stirred continuously and maintained at an alkaline pH (pink to phenolphthalein added as an internal indicator) by the addition of N- or 4N-NaOH at intervals during the addition of the $POCl_3$ and for some time afterwards. When no more NaOH was required, the aqueous layer was dialyzed at about 5° in cellophan sacs against 0·9% (w/v) NaCl (to which a little toluene had been added) until free from inorganic phosphate. In some of the earlier experiments distilled water was used as the dialyzing fluid. To determine the extent of the phosphorylation, total P and total N determinations were made on the original solution and on samples of the phosphorylated proteins precipitated from the solution in various ways. The results are usually expressed as a ratio (wt. of P/wt. of N).

Precipitation of the phosphorylated proteins. In most of the experiments the phosphorylated protein was precipitated from solution by adjusting the pH to 4–4·6 with 0·1N-HCl, using external indicators; the best results were obtained by using 2% (v/v) acetic acid for final adjustment of the pH to give maximal precipitation. Precipitation was least satisfactory and sometimes impossible with phosphorylated proteins of low P/N ratios. In several experiments the proteins were precipitated by the addition of 4 vol. of a 10% (w/v) solution of trichloroacetic acid (subsequently referred to as TCA). When these precipitates were washed with ethanol or acetone to remove TCA, some protein dissolved, but this was precipitated again by the addition of N-NaOH and a few drops of conc. $MgCl_2$ solution. The unwashed precipitates obtained with TCA, when dried over $CaCl_2$, had a horny texture. In other experiments the phosphorylated protein was precipitated with 6 vol. of ethanol, since the precipitates obtained with TCA gave lower P/N ratios than did those obtained with HCl; it was thought possible that some of the less firmly attached phosphate groups had been removed from the protein by treatment with TCA. The P/N ratios of ethanol-precipitated phosphorylated proteins were almost always the same as those for the HCl-precipitated product from the same solution, where this solution was free from inorganic phosphate. Where, however, the solution contained inorganic phosphate, the ethanol-precipitated proteins gave a relatively high P/N ratio, and tests made with ^{32}P containing inorganic phosphate showed that this was due to the precipitation of appreciable amounts of inorganic phosphate by the ethanol. The results obtained with ethanol-precipitated products have therefore been discarded, except where they refer to precipitates obtained from solutions free from inorganic phosphate.

P *determinations.* These were made by King's (1932) modification of the Fiske & Subbarow (1925) method.

Preparation of phosphorylated proteins for immunization

'High P' proteins. Sterile human serum (110 ml.) was phosphorylated in two equal batches, using a $POCl_3$/protein ratio of 0·5/1. After dialysis, the phosphorylated proteins were precipitated by acid (N-HCl and 2% acetic acid) and redissolved in sterile 0·9% NaCl with the addition of a little N-NaOH. Phenol (final concentration 0·25%) was also added. This solution had a protein content of 5·1% (w/v) and a P/N ratio of 0·14.

'Low P' proteins. Sterile human serum (165 ml.) was phosphorylated, dialyzed, precipitated and redissolved as above. The final solution contained 7·25% protein (P/N ratio 0·14) and 0·25% phenol. A mixture of this solution and 0·25 vol. of 0·75M-$NaHCO_3$-H_2CO_3 buffer (pH 7·5) was placed in a stoppered bottle, the air in the bottle displaced by CO_2 and the bottle sealed and kept at 39° for 139 hr. The solution was then dialyzed against cold 0·9% NaCl to remove any free phosphate which had been split off at 39°, and 0·25% of phenol added. This solution contained 5·2% protein with a P/N ratio of 0·02.

Preparation of antigens for precipitin tests. Rabbit plasma and chicken serum were phosphorylated using a $POCl_3$/protein ratio of 0·5/1. The phosphorylated rabbit plasma had a P/N ratio of 0·17; some of this solution was kept at 37° with an equal volume of 0·75M-$NaHCO_3$-H_2CO_3 buffer (pH 7·5) for 4 days, followed by dialysis ('low P' rabbit proteins). In an attempt to obtain a phosphorylated antigen having a higher P/N ratio, rabbit serum was phosphorylated using a $POCl_3$/protein ratio of 2·5/1, but the product had the same P/N value (0·17) as the phosphorylated rabbit plasma preparation described above. Gelatin solutions (0·25 and 0·5%) were treated with different amounts of $POCl_3$ ($POCl_3$/protein ratios of 0·5 and 2·5/1 respectively). The amount of protein present after dialysis was, however, very small, and in both cases there was insufficient material for accurate P determinations.

Immunization. The 'high P' and 'low P' phosphorylated human serum proteins were each injected into a group of five rabbits. Two rabbits of each group received an initial intravenous injection of 4·5 or 5 ml. of the solution, followed by five or six intraperitoneal injections of 5 ml. (rising gradually to 10 ml.) at intervals of 7–10 days. Sera from these rabbits were tested after the third and subsequent injections. The remaining three rabbits of each group received three courses of intraperitoneal injections; each course consisted of four or five injections (each of 1·8 or 2·0 ml. of 5% protein solution) at intervals of 2–4 days, with an interval of 8–12 days between the courses. Sera for precipitin tests were collected after the second and third courses.

Precipitin tests. The tests were made as described previously (Hopkins & Wormall, 1933). The concentration of antigen in the Tables refers to dilution (with 0·9% NaCl) of a solution of the antigen containing approx. 5% protein.

RESULTS

Phosphorylation of 'dephosphorized casein', serum globulin and serum albumin

'Dephosphorized casein' was prepared from 'soluble casein' (British Drug Houses Ltd.) by the action of 0·25N-NaOH (Plimmer & Bayliss, 1906; Rimington & Kay, 1926), the product being purified by two precipitations. The globulin and albumin were prepared from human serum by half and full saturation with $(NH_4)_2SO_4$; they were purified by repeated precipitation, and were finally dialyzed against 0·9% NaCl until free from $(NH_4)_2SO_4$. Sufficient 0·9% NaCl was added to each solution to give a protein concentration of 4·8% (w/v).

Each solution (20·8 ml. of dephosphorylated casein, globulin or albumin) was phosphorylated with 0·5 g. of $POCl_3$ (i.e. a $POCl_3$/protein ratio of 0·5/1), as described above. During the course of phosphorylation the globulin and dephosphorized casein solutions became gelatinous, and adequate stirring was difficult; 0·9% NaCl, equal to about 0·25 of the total volume of the solution, was therefore added before the phosphorylation had been completed. The albumin solution was similarly diluted before phosphorylation. After dialysis the phosphorylated proteins were repeatedly precipitated with HCl, the precipitates being washed with distilled water to which a few drops of acetic acid had been added. P/N ratios, determined on the precipitates obtained in the 4th, 5th and 6th precipitations are given in Table 1.

The results showed that, under these conditions, phosphorylation of serum albumin, serum globulin and dephosphorized casein with $POCl_3$ readily effects the introduction of one P atom for every 33–38 N atoms in the protein molecule; this represents a P content of 1·0–1·07% for the phosphorylated proteins, calculated on the basis of 16% N in the protein. It is interesting to note that approximately the same amount of P was introduced into three different types of protein. The results also show that after three precipitations of these phosphorylated proteins with HCl, further acid precipitation yielded products of approximately constant P content.

Table 1. P *contents of 'rephosphorized' casein and phosphorylated human serum albumin and globulin*

	P/N (weight ratios)		
Precipitation	Phosphorylated albumin	Phosphorylated globulin	Rephosphorized casein
4th	0·062	0·067	0·063
5th	0·059	0·070	0·062
6th	0·057	0·065	0·060

Conditions of preparation of phosphorylated human serum proteins

Effect of varying the $POCl_3$/protein ratios and other experimental conditions. Since it was our intention to introduce ^{32}P into proteins for immunological investigations several experiments have been carried out to determine the amount of P which enters the protein when it is treated with amounts of $POCl_3$ considerably less than those used by Rimington (1927) and other investigators. In a typical experiment, three samples, each of 14·3 ml. of pooled human serum and containing 1 g. of protein, were treated with 0·5, 0·3 and 0·1 g. of $POCl_3$ respectively. The P/N ratios of the dialyzed solutions and of the acid-precipitated products from the 1st, 2nd and 3rd precipitations showed that with the smaller amounts of $POCl_3$ less P was introduced into the

Table 2. *Comparison of* P/N *ratios of human serum proteins phosphorylated under varying conditions*

Material	Protein content (%)	Ratio $POCl_3$/protein	P/N ratio: Dialyzed solution	P/N ratio: HCl precipitate 1st	2nd	3rd
Human serum	7·25	0·5	0·104	0·111	0·100	0·101
		0·3	0·078	0·083	0·074	0·071
		0·1	0·030	0·033	0·031	0·033
Freeze-dried human serum	7·0	0·3	0·075	—	—	—
	12·5	0·3	0·095	—	—	—

protein (Table 2). With $POCl_3$/protein ratios of 0·5/1, 0·3/1 and 0·1/1, the resulting phosphorylated proteins contained 1·62, 1·14 and 0·53 % of P respectively, calculated on a 16 % N content for the protein. This would correspond respectively to the introduction of about 52, 37 and 17 P atoms into each protein molecule, assuming a mean mol. wt. of 10^5 for the serum proteins.

Phosphorylation of a concentrated solution of human serum proteins. It was thought possible that less $POCl_3$ would be lost by hydrolysis if the solution to be phosphorylated had a higher protein content. This point was tested using a concentrated solution of serum proteins and a relatively low $POCl_3$/protein ratio. Samples of freeze-dried human serum (kindly presented by Dr R. R. Race) were dissolved in water to give 12·5 and 7 % protein solutions. These solutions were phosphorylated with sufficient $POCl_3$ to give a $POCl_3$/protein ratio of 0·3/1 and then dialyzed. The results recorded in the lower part of Table 2 showed that a saving of $POCl_3$ could be effected by the use of the more concentrated protein solution, but this method was not used in later experiments since it gave a greater amount of insoluble material than did phosphorylation of normal serum.

Stability of the phosphoryl group attached to proteins

Preliminary investigations had suggested that loss of phosphoryl groups occurred when the phosphorylated proteins were kept at 37°, even at or near neutrality, and the following experiments were therefore carried out to determine to what extent this loss might occur under physiological conditions, e.g. after injection into the animal body.

Loss of P *from phosphorylated human serum proteins, 'rephosphorized' casein and casein at* 37°. Solutions of phosphorylated human serum proteins, casein, and in one case rephosphorized casein (all phosphorylated with a $POCl_3$/protein ratio of 0·5/1 and precipitated by HCl), were treated with equal volumes of either 0·75 M-$NaHCO_3$-H_2CO_3 buffer (pH 7·5) or veronal buffer (pH 6·8) and the mixtures sealed in bottles with a little toluene, and kept at 37°. Samples were taken at intervals and the proteins precipitated by TCA. The results (Table 3) showed that the phosphorylated human serum proteins had lost appreciable amounts of combined P when the solutions (at pH 6·8 or 7·5) were kept at 37°; at least one fifth of the combined P in 24 hr. and well over half in 6 days. Somewhat similar results were obtained in an experiment with rephosphorized casein (at pH 6·8). The P of casein is, however, much less labile under these conditions, and the loss in 6 days was usually less than 10 %. In another experiment the incubation at pH 7·5 was continued for 20 days, with results similar to those recorded in Table 3; analysis of the TCA-precipitated proteins showed that the phosphorylated serum proteins had lost about half their combined P during the incubation, whereas casein had lost little or none.

Table 3. P *content of phosphorylated proteins kept at* 37° *and* pH 6·8 *or* 7·5

Exp.	pH	Duration of incubation (hr.)	P/N ratio of TCA-precipitated proteins: Phosphorylated human serum proteins	Rephosphorized casein	Casein*
1	7·5	0	0·051	—	0·052
		24	0·041	—	0·048
		72	0·027	—	0·047
		147	0·024	—	—
	6·8	0	0·067	0·042	0·048
		24	0·049	0·039	0·047
		72	0·027	0·025	0·046
		147	0·022	0·021	—
2	7·5	0	0·041	—	0·042
		142	0·018	—	0·039
		306	0·020	—	0·036

* The casein separated out from both buffer mixtures during the incubation, but samples were taken from the suspension after shaking.

Loss at 37° of P from phosphorylated serum proteins prepared with different $POCl_3$/protein ratios. Different preparations of phosphorylated serum proteins showed differences in the rate of loss of combined P when the solutions were kept at 37° and it was thought that there might be qualitative differences in this respect between the phosphate groups most readily attached to the protein (i.e. by the use of lesser quantities of $POCl_3$) and those attached by more drastic phosphorylation.

Human serum (20 ml.) was phosphorylated with 0·14 g. $POCl_3$ (i.e. a $POCl_3$/protein ratio of 0·1/1). The product was dialyzed against distilled water for 5 hr., and then against 0·9% NaCl until free from inorganic phosphate. The solution was brought to pH 7·5 with 0·2N-NaOH and added to an equal volume of 0·75M-$NaHCO_3$-H_2CO_3 buffer. Toluene was added and the mixture was kept in a sealed tube at 37°, samples being withdrawn after 141 and 260 hr. for precipitation of the proteins with TCA.

The results (Table 4) showed that even with the 'lightly phosphorylated' proteins there was fairly rapid loss of P when the solution was kept at 37°.

Table 4. *Loss at 37° and pH 7·5 of P from phosphorylated human serum proteins prepared with a $POCl_3$/protein ratio of 0·1/1*

Duration of incubation (hr.)	Type of precipitation*	P/N ratio
0	(Dialyzed liquid)	0·042
	HCl + acetone	0·040
	TCA	0·021
141	TCA	0·005
260	TCA	0·004

* The precipitates obtained with TCA were washed with ethanol and acetone.

A further experiment was made to cover a greater range of $POCl_3$/protein ratios, viz. 0·025–0·05/1. All these resulting phosphorylated human serum proteins, with P/N ratios ranging from 0·017 to 0·095, lost 80% of their combined P when the solutions were kept at 37° for 142 hr. (Table 5). The possibility that some of the phosphorylated protein was split up into smaller molecules of P-containing organic substances during the incubation, and that these smaller molecules subsequently passed through the dialyzing sac, was not explored.

Table 5. *Loss of P at 37° and pH 7·5 from phosphorylated human serum proteins prepared with different $POCl_3$/protein ratios*

$POCl_3$/protein ratio used in the preparation	P/N ratio: Dialyzed* solution	P/N ratio: After 142 hr. at 37° and subsequent dialysis*
0·025	0·017	0·005
0·05	0·024	0·005
0·10	0·038	0·008
0·20	0·055	0·010
0·30	0·076	0·015
0·50	0·095	0·023

* Dialysis at 4–6°.

Immunization with phosphorylated serum proteins

Two different antigens were used for immunization in the hope that antisera of differing serological specificity might be obtained. The 'high P' phosphorylated serum protein was prepared by the action of the usual great excess of $POCl_3$ on human serum proteins, and the 'low P' protein was obtained by incubating a solution of the 'high P' proteins at 39° and pH 7·5, with subsequent dialysis to remove free phosphate; incubation for 6 days removed about 6/7 of the combined P of the 'high P' phosphorylated proteins.

Precipitin tests carried out with the sera of the injected rabbits over a long period of immunization showed that all the rabbits had produced antibodies capable of reacting strongly with the antigens used for the injections (phosphorylated human serum proteins), with no marked difference between the results with the 'high P' and those with the 'low P' proteins. The antisera also gave strong or very strong precipitin reactions with untreated human serum, showing that phosphorylation had not destroyed the species specificity of the serum proteins. In a few cases only, the antisera reacted slightly with phosphorylated rabbit serum proteins, but no precipitate was obtained with phosphorylated chicken serum proteins or with phosphorylated gelatin. The results of some typical precipitin tests are given in Table 6.

DISCUSSION

The phosphorylated proteins obtained by the action of $POCl_3$ on serum proteins are readily precipitated by dilute acid (at pH 4–5 for the strongly phosphorylated proteins). In many other respects also, phosphorylation effects a distinct change in properties. With a moderate excess of phosphoryl chloride, the amount of P introduced into the proteins under the conditions of our experiments may correspond to as much as 50 phosphoryl groups/mol. of protein, assuming a mean mol. wt. of 10^5 for these proteins. Heidelberger *et al.* (1941), in similar investigations, found that crystalline egg albumin could take up 20–30 phosphoryl groups/mol. on the basis of a mol. wt. of 36,000 for egg albumin, and they noted that 'the introduction of these polar groups is accompanied by a remarkable series of changes in the chemical, physical and immunological properties of the protein'.

Table 6. *Precipitin reactions with antisera to phosphorylated human serum proteins*

			Antisera to phosphorylated human serum proteins			
			'Low P'		'High P'	
Antigen		Dilution of antigen	Rabbit no. 461	Rabbit no. 463	Rabbit no. 480	Rabbit no. 482
Human serum		1 : 20	+	+±	±	++
		1 : 100	+++±	+++±	+	+++±
		1 : 500	++	+±	±	+
		1 : 2500	±	tr	tr	+
Phosphorylated human serum proteins	'Low P'	1 : 20	+±	+±	tr	.
		1 : 100	+++±	+++±	+	.
		1 : 500	+±	+±	tr	.
		1 : 2500	±	tr	tr	.
	'High P'	1 : 20	+	.	tr	+±
		1 : 100	+++±	.	+	+++±
		1 : 500	+±	.	±	+++±
		1 : 2500	±	.	tr	+
Phosphorylated rabbit serum proteins	(*a*)*	1 : 20	–	–	–	–
		1 : 100	–	–	–	–
		1 : 500	–	–	–	±
		1 : 2500	–	–	–	–
	(*b*)*	1 : 20	–	–	–	–
		1 : 100	–	–	–	f.tr
		1 : 500	–	–	–	tr
		1 : 2500	–	–	–	±

* The phosphorylated rabbit serum preparations (*a*) and (*b*) were obtained by the use of a $POCl_3$/protein ratio of 0·5/1 and 2·5/1 respectively; both products were dialyzed free from inorganic phosphate.

Precipitin readings: – (no precipitate); f.tr (faint trace); tr (trace); ±, +, +±; ++, etc. in increasing degrees of precipitation.

In most of the experiments described in this paper we have used phosphorylated mixed serum proteins, the chief object being to prepare antigenic proteins containing ^{32}P, and a mixture of the phosphorylated serum globulins and albumins seemed most suitable for many of the serological tests. For some of the experiments reported here, however, and for others to be described later, purified and less complex mixtures of phosphorylated proteins have been used. Another difference between our experiments and those of Heidelberger *et al.* (1941) is that in nearly all cases we have used considerably less phosphoryl chloride than was used by them, or by Rimington (1927). Our object was to ensure minimum loss of valuable ^{32}P in parallel experiments with 'radio' $POCl_3$. Even with this smaller amount of phosphoryl chloride (0·5 g./g. of protein as compared with the 2–3 g./g. of protein used by Heidelberger *et al.* and by Rimington in most of their experiments) the proteins were phosphorylated to a comparable degree: for example, 1–1·5 % of P, as compared with 1·52 % for heavily phosphorylated egg albumin (Heidelberger *et al.* 1941), and 0·71 % for phosphorylated denatured serum globulin (Rimington, 1927). Furthermore, in one of our experiments it was found that a fivefold increase in the amount of phosphoryl chloride used did not lead to any significant increase in the amount of P attached to rabbit serum proteins.

The amount of P introduced into the serum proteins varied somewhat from one preparation to another, and the reason for this variation is not known. Freshly distilled $POCl_3$ was used in practically all our experiments, and the technique of phosphorylation was kept as constant as possible. Different samples of human serum may differ in their capacity to react with $POCl_3$, but it is difficult to understand why, for example, a P/N ratio of 0·06–0·07 was obtained for the phosphorylated human serum albumin and globulin in one experiment (Table 1), whereas most subsequent experiments with serum proteins, and involving the use of the same relative amount of phosphoryl chloride, gave products with a P/N ratio of approximately 0·10. Similar variations were also noted by Heidelberger *et al.* (1941): one of their phosphorylated egg albumin preparations contained 2·71 % of P and had a P/N ratio of 0·18, as against P/N ratios of 0·104–0·115 for other preparations obtained by the same method of phosphorylation. It seems probable, as they suggest, that part of the P of phosphorylated proteins with a high P/N ratio is in a very labile form, and that variable amounts are split off when the experimental technique for isolating the phosphorylated protein is varied even slightly. We, like they, have found that this lability is very marked at 37°.

Of the various protein precipitants which we have used for the separation of the phosphorylated proteins, dilute acid (HCl, followed by final adjustment of the pH with acetic acid) has proved most satisfactory. Trichloroacetic acid was less satisfactory, and it is possible that the high acidity caused by this reagent (about pH 0·6 in the experiments referred to in Table 3) may have resulted in appreciable removal of P from the phosphorylated proteins. Where a solution of phosphorylated protein was required for immunological purposes, purification was effected by dialysis, followed by repeated acid precipitation where the preparation was required for injection.

The results of the immunological experiments showed that the antibodies produced by the injection of phosphorylated human serum proteins were not specific for the phosphoryl groups of the protein, for the antisera obtained gave little or no reaction with phosphorylated rabbit or chicken serum proteins. This result was rather surprising, for the introduction of an appreciable number of polar groups into the molecule of an antigenic protein usually leads to the acquisition of a new serological specificity characteristic for the introduced groupings. It is probable, however, that the phosphorylated human serum proteins rapidly lost most of their combined P after injection into rabbits; there was certainly no lack of antigenic power in the injected material, since powerful antibodies against the injected antigen (phosphorylated human serum proteins) were formed. This view is supported by the *in vitro* lability of the combined phosphate groups at 37° and pH 7·5 (Tables 3–5); incubation for several days resulted in a very marked decrease, sometimes as much as 80 %, in the combined P of the proteins precipitable by trichloroacetic acid or those freed from dialyzable P-containing compounds.

If the injected phosphorylated proteins rapidly lost their combined P, as the experiments described in this paper and in the related investigations with ^{32}P-containing phosphorylated proteins (Banks, Boursnell, Dewey, Francis, Tupper & Wormall, 1948) suggest, the failure of the injected rabbits to produce antibodies to phosphorylated heterologous protein, and the production of antibodies capable of reacting with untreated human serum proteins, can readily be explained. It seems probable that our phosphorylated human serum protein preparations differed considerably, in their *in vivo* stability, from the phosphorylated egg albumin of Heidelberger *et al.* (1941), for the antisera produced by the injection of the latter antigen reacted only slightly with unchanged egg albumin. Heidelberger *et al.* obtained specific antibodies against phosphorylated egg albumin, but they observed that the new specificity conferred on egg albumin by the introduction of phosphoryl groups is not entirely a haptene specificity in the sense defined by Landsteiner (1936).

The possibility of an enzymic removal of P from phosphorylated proteins, either by an enzyme present in our preparations of phosphorylated human serum proteins or in rabbit blood, cannot be excluded, but this possibility has not as yet been investigated. We intend, however, to study the *in vivo* stability of phosphorylated human serum proteins, phosphorylated egg albumin and other phosphorylated proteins. It seems probable from the evidence already available that phosphorylated human serum proteins may not be the most suitable proteins for our investigations with ^{32}P-containing protein antigens. For some of these purposes vitellin is more satisfactory (Francis & Wormall, 1948), but unfortunately it is almost insoluble in water when freed from firmly combined phosphatide.

The action of phosphoryl chloride on proteins may concern several groups of the protein, e.g. the hydroxy groups of serine and threonine, and possibly the phenol group of tyrosine, the free amino group of lysine, and the guanidine group of arginine. Some of the attached phosphoryl groupings will, of course, be less firmly attached than others, and the existence of different P-protein linkages might readily explain the considerable variations in the rate with which P is removed from phosphorylated proteins. It is difficult to understand, however, why this process is so rapid, particularly at 37°, in solutions kept at pH 7·5, for some phosphorylated amino-acids are quite stable. Plimmer (1941), for example, found that the phosphoric esters of tyrosine, hydroxyproline, threonine, serine and *iso*serine were all stable to 0·5N- or even N-NaOH at 37°. It is our intention to study this problem further, with the aid of serological methods.

SUMMARY

1. Human serum proteins have been phosphorylated by phosphoryl chloride, and some of the chemical and immunological properties of the products have been studied.

2. These phosphorylated proteins lost appreciable amounts of their combined P when they were kept in solution at 37° and pH 7·5 or 6·8.

3. Immunization of rabbits with phosphorylated human serum proteins produced antibodies which reacted strongly with the injected proteins and also with untreated human serum proteins.

4. These antisera gave little or no precipitation with phosphorylated rabbit serum proteins or phosphorylated chicken serum proteins. No evidence, therefore, was obtained of the formation of antibodies specific for the phosphoryl group of phosphorylated proteins. It is probable, however, that the phosphorylated serum proteins rapidly lost a considerable fraction of their combined P after injection into the rabbits.

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POSTSCRIPT. Since this paper was in the press we have seen the very valuable account of the chemical and immunological properties of phosphorylated crystalline horse serum albumin given by Mayer & Heidelberger (1946). We hope to make full reference to this work later.

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Studies in the Biochemistry of Micro-organisms

77. A SURVEY OF FUNGAL METABOLISM OF INORGANIC SULPHATES

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Clutterbuck, Mukhopadhyay, Oxford & Raistrick (1940), in a communication from this laboratory, described the results of a quantitative survey of the chlorine metabolism of 139 species or strains of moulds grown on Czapek-Dox 5% glucose solution containing 0·5 g. KCl/l. as the sole source of chlorine.

The results are now given of a parallel quantitative survey of the sulphur metabolism of 115 species or strains of moulds grown on the same medium containing 0·5 g. $MgSO_4.7H_2O$ and 0·01 g. $FeSO_4.7H_2O$/l. as the sole source of sulphur.

EXPERIMENTAL

Cultural conditions

The medium used throughout this investigation was the well-known Czapek-Dox solution of the following composition: glucose, 50 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; $FeSO_4.7H_2O$, 0·01 g.; distilled water, 1 l. All the constituents of the medium were of analytical reagent purity. Hence the sole source of sulphur was present as the sulphates of magnesium and iron, and amounted to 22·9 mg./flask (6·6 mg./100 ml.) at the time of sowing. This medium (350 ml.) was accurately measured into each of a number of 1 l. conical flasks plugged with cotton wool, and sterilized by steaming for 0·5 hr. on each of 3 consecutive days. The flasks were then inoculated with a pure culture of the chosen species of mould, and incubated at 24° for the requisite period of days.

The sulphate content of duplicate uninoculated flasks from each batch was determined by the method described later. The inoculated flasks were treated as follows. The mycelium from each species was separated quantitatively by filtration through a Buchner funnel, and was thoroughly washed with warm water. It was then dried to constant weight *in vacuo*, weighed, ground and stored in a closely stoppered bottle prior to examination. The culture filtrate and washings were made up to a total volume of 500 ml., and analyzed by the methods described in the next section.

Analytical methods

I. *Sulphur as inorganic sulphate in culture filtrate*

The concentration of inorganic sulphate present in the uninoculated medium is quite low (equiv. to 22·9 mg. S/350 ml., or 4·58 mg./100 ml. after dilution to 500 ml.). The medium also contains relatively considerable amounts of nitrate, phosphate and chloride. Further, the culture filtrate resulting from the growth of different moulds may contain metabolic products of complex and unknown nature and may vary from almost colourless to deeply coloured. After a considerable amount of exploratory work, details of which will be published elsewhere, on a number of published methods the one finally chosen as being the most generally useful for our purpose was that based on Øllgaard's (1934) method. This method involves the precipitation of inorganic sulphate with benzidine in acetone and titration of the precipitate, after liberation of the sulphate with a little NaOH, with a standard $BaCl_2$ solution using sodium rhodizonate as indicator. Using this method we found that, in solutions containing 0·3 mg. of sulphate S/10 ml. portion, a precision of $\pm 1\%$ in duplicates can in general be assured, and of $\pm 5\%$ with certainty.

Reagents

Trichloroacetic acid. 20% (w/v) solution in water.

Benzidine. 1% solution in pure acetone, freshly prepared each day.

Sodium hydroxide. N-NaOH, in water.

Ethanolic magnesia mixture. NH_4Cl (6 g.) and $MgCl_2.6H_2O$ (6 g.) in water (100 ml.) and made up to 1 l. with absolute ethanol.

Barium chloride. 0·02 N-$BaCl_2$, in water.

Indicator. A freshly prepared aqueous solution of sodium rhodizonate of a fairly deep orange colour.

All reagents should be A.R.

Method. The solution (20 ml.) to be examined is acidified with trichloroacetic acid (10 ml.). Any precipitate formed is separated by filtration. Duplicate 10 ml. samples of the clear solution are pipetted into 50 ml. centrifuge tubes. Benzidine solution in acetone (25 ml.) is added to each tube in a fine stream, and the tubes are capped and allowed to stand for at least 0·5 hr. They are then centrifuged at medium speed for 15–20 min., and the clear supernatant fluid is carefully decanted. The tubes are now inverted on filter paper to drain residual acetone. It was found by control experiments that washing of the precipitate with acetone is unnecessary, and that even when the culture filtrate is highly coloured the precipitate left after decantation is almost white, and the amount of residual colouring matter was never sufficient to interfere with subsequent titration. The precipitate is now prepared for titration by dissolving in 0·1 ml. of N-NaOH. Shaking and warming facilitate solution. Ethanolic magnesia mixture (10 ml.) is added to the solution to precipitate phosphates, and the tubes are kept at a temperature of 65–70° in a thermostat during the titration. Sufficient sodium rhodizonate solution is added to give a definite yellow colour to the solution, and care must be taken to add the same amount of indicator to each tube of a series of estimations. 0·02 N-$BaCl_2$ is added drop by drop from a micro-burette graduated in 0·01 ml. During the titration the contents of the tube are rotated gently. The yellow colour changes through orange and reddish orange to a rather bright red. The $BaCl_2$ should be added carefully until there is a definite and *permanent* blue tint in the red, continuing heating in the thermostat and further addition of $BaCl_2$ if necessary. Illumination needs to be good; a blue-glass electric light placed close to the tube and a white background help considerably.

1 ml. of 0·02 N-$BaCl_2$ = 0·3207 mg. S.

Of the 119 species and strains of moulds examined only four, which were discarded, gave trouble and then only after a very long period of incubation. In three cases, i.e. *Penicillium frequentans* Westling, P. 28; *P. luteum* Zukal, P. 42; *P. rugulosum* Thom, P. 50, the interference was due to the extreme viscosity of the culture filtrate, and in the fourth, *Aspergillus niger* van Tieghem, no. 33, a heavy precipitate prevented titration.

II. *Sulphur as total inorganic sulphate in culture filtrate after heating with* 0·3 N-HCl

The method commonly used for the hydrolysis of the ethereal sulphates in urine has been modified and applied to the mould culture filtrates. A portion of the culture filtrate is accurately measured into a conical flask and acidified with $\frac{1}{12}$ vol. pure conc. HCl, so that the final concentration of HCl is approx. 0·3 N. A small funnel and watch glass are inserted in the neck of the flask to lessen evaporation, and its contents are boiled gently for 30 min. After cooling, the solution is transferred to a measuring flask, a little bromocresol green is added and the solution neutralized with NaOH. The total inorganic sulphate now present is determined in portions by the method described in § I. Reagents are checked by means of a blank experiment. Phenyl potassium sulphate, initially free from inorganic sulphates, gave a 96·2% yield of inorganic sulphate when estimated by this method.

III. *Sulphur as inorganic sulphate generated in culture filtrate after heating with* 0·3 N-HCl

This is obtained by subtracting the value given by the estimation in § I from that obtained as described in § II.

IV. *Total sulphur in culture filtrate*

To a sample of the culture filtrate (usually 100 ml.) is added fuming HNO_3 (10 ml.) and KNO_3 (0·5 g.). The mixture is evaporated almost to dryness in a Kjeldahl flask, and is then treated exactly as in the estimation of total sulphur in the mycelium described in § VI.

V. *Sulphur in culture filtrate not present as inorganic sulphate after heating with* 0·3 N-HCl

This is obtained by subtracting the value given by the estimation in § II from that obtained as described in § IV.

VI. *Total sulphur in the mycelium*

About 1 g. of dried and powdered mycelium is accurately weighed, and is gently digested for 4 hr. in a Kjeldahl flask with fuming HNO_3 (10 ml.) and KNO_3 (0·5 g.). The acid is then removed in a stream of air on a water bath. A further 10 ml. fuming HNO_3 are added, the contents heated for 2 hr. and the acid removed as before. The residue is now digested on a sand bath for 1–2 hr. with H_2O_2 (10 ml. of 100 vol.) added at intervals 2 ml. at a time. The clear solution is evaporated on the water bath, taken up in 0·25 N-HCl (20 ml.) and evaporated to dryness. Evaporation with HCl is repeated, and the final white residue is dissolved in 100 ml. of a mixture of water and trichloroacetic acid (final acid concentration, 6%). The amount of inorganic sulphate is estimated in 10 ml. samples by precipitation with benzidine as described in § I.

VII. *Residual glucose in the culture filtrate*

This is estimated polarimetrically.

RESULTS

The utilization of sulphur initially present as inorganic sulphate, by 115 *mould species or strains*

In this part of the investigation only the culture filtrate was examined.

Two flasks, each containing 350 ml. of Czapek-Dox culture medium with a sulphate content equivalent to 22·9 mg. of sulphur/flask, were inoculated at the same time and from the same culture of the species or strain of mould being examined. One flask was, in general, harvested after an incubation period of 17–25 days, after which time the residual glucose was usually between 0·1 and 1·0%. The second flask was harvested after a much longer incubation period, usually 5–10 weeks, by which time the glucose had either been completely metabolized, or its utilization had become extremely slow. The residual inorganic sulphate was estimated in each flask by the method described above.

The results obtained are given in Table 1. The figures given in column 5 show the percentage utilization of sulphate, so that a figure of 97·6% means that only 2·4% of the sulphur originally present in the culture medium as inorganic sulphate remains in that form in the culture filtrate. The moulds examined are listed in descending order of

Table 1. *Utilization of inorganic sulphate by 115 species and strains of fungi*

Organism	Catalogue no.*	Incubation period (days)	Residual glucose by polarimeter (g./100 ml.)	Utilization of SO_4 (%)
	Species of *Penicillium*			
P. puberulum Bainier	P. 47	24	0·2	97·6
		38	0·0	96·0
P. notatum Westling	P. 45	23	0·7	95·6
		38	0·0	96·4
P. citrinum Thom	P. 27	21	0·6	85·3
		39	0·1	70·3
P. olivino-viride Biourge	P. 46	24	0·2	80·6
		37	0·2	97·4
P. brunneo-rubrum Dierckx	P. 8	21	0·4	80·3
		58	0·0	58·0
P. lanosum Westling	P. 86	21	1·0	80·2
		68	0·0	59·6
P. candido-fulvum Dierckx	P. 12	18	3·6	79·6
		65	0·1	72·0
P. chrysogenum Thom	P. 57	25	0·0	79·1
		39	0·0	40·6
P. phaeo-janthinellum Biourge	P. 91	22	0·1	76·6
		56	0·0	60·4
P. griseo-brunneum Biourge	P. 36	22	0·2	76·1
		37	0·0	45·8
P. Hagemi Zaleski	P. 84	21	0·3	74·0
		68	0·0	55·8
P. citreo-nigrum Dierckx	P. 20	21	1·2	73·4
		57	0·0	96·6
P. citrinum Thom	P. 26	24	0·1	73·4
		56	0·0	55·7
P. digitatum Saccardo	P. 33	43	0·0	73·0
		66	0·5	79·7
P. aurantio-brunneum Dierckx	P. 2	20	0·8	68·8
		60	0·0	95·2
P. citreo-viride Biourge	P. 24	24	0·3	68·8
		55	0·0	68·4
P. commune Thom	P. 30	21	0·4	67·8
		38	0·0	50·7
P. patris-mei Zaleski	P. 90	22	0·3	66·6
		68	0·0	50·6
P. solitum Westling	P. 52	24	0·6	66·1
		39	0·1	60·9
P. caseicolum Bainier	P. 11	18	2·0	66·0
		60	0·0	33·9
P. citreo-roseum Dierckx	P. 22	21	0·7	65·5
		57	0·0	98·1
P. chloro-leucon Biourge	P. 14	18	0·4	64·8
		55	0·1	52·8
P. chloro-phaeum Biourge	P. 15	18	1·4	64·8
		57	0·0	76·5
P. ochraceum Bainier	P. 88	22	0·4	63·5
		68	0·0	47·2
P. baculatum Westling	P. 7	21	0·3	63·4
		59	0·0	58·0
P. chrysitis Biourge	P. 16	18	3·1	60·5
		61	0·0	58·4
P. griseo-roseum Dierckx	P. 39	22	0·8	60·4
		39	0·2	38·9
P. flavido-marginatum Biourge	P. 78	20	0·1	59·8
		57	0·1	26·2

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Table 1 (*cont.*).

Organism	Catalogue no.*	Incubation period (days)	Residual glucose by polarimeter (g./100 ml.)	Utilization of SO_4 (%)
P. Bialowiezense Zaleski	P. 71	19	0·2	59·7
		56	0·0	35·9
P. citreo-roseum Dierckx	P. 23	24	0·4	59·2
		55	0·1	61·1
P. citrinum Thom	P. 6	20	1·1	58·5
		60	0·0	58·4
P. fusco-glaucum Biourge	P. 81	21	0·5	58·1
		56	0·0	82·3
P. chrysogenum Thom	P. 19	21	0·4	57·7
		55	0·0	60·5
P. frequentans Westling	P. 29	21	0·8	56·3
		39	0·0	80·1
P. griseo-fulvum Dierckx	P. 38	22	2·0	54·1
		43	0·7	48·9
P. citreo-nigrum Dierckx	P. 21	21	2·2	53·7
		61	0·2	56·8
P. Raciborskii Zaleski	P. 92	22	0·1	52·8
		67	0·0	42·8
P. stilton Biourge	P. 54	25	2·0	52·8
		64	0·1	62·8
P. citreo-sulfuratum Biourge	P. 66	18	2·3	50·7
		71	0·0	66·2
P. expansum Link.	P. 41	23	1·1	50·2
		43	0·1	95·8
P. biforme Thom	P. 72	19	0·1	49·8
		67	0·1	24·5
P. citreo-sulfuratum Biourge	P. 59	26	1·7	49·7
		65	0·1	52·0
P. cinerascens Biourge	P. 65	18	2·8	48·5
		71	1·0	52·0
P. spinulosum Thom	P. 96	25	0·1	48·0
		67	0·0	39·8
P. citrinum Thom	P. 25	24	0·3	47·4
		55	0·0	65·6
P. cyaneo-fulvum Biourge	P. 34	22	1·2	45·4
		66	0·5	55·0
P. Oledzkii Zaleski	P. 89	22	2·4	44·5
		69	0·0	60·9
P. Siemaszki Zaleski	P. 95	25	1·0	44·1
		69	0·1	31·0
P. sublateritium Biourge	P. 55	25	0·3	42·8
		42	0·0	60·9
P. purpurogenum Stoll	P. 48	24	0·3	40·6
		39	0·1	45·4
P. viridi-dorsum Biourge	P. 56	25	1·3	40·6
		42	0·1	43·7
P. fellutanum Biourge	P. 67	19	0·5	40·2
		56	0·0	48·5
P. Dierckxii Biourge	P. 32	22	2·0	39·7
		43	0·1	61·2
P. roqueforti Thom	P. 93	22	3·1	37·6
		69	0·0	40·6
P. cyaneo-fulvum Biourge	P. 31	21	1·5	37·5
		65	0·5	50·7
P. aurantio-griseum Dierckx var. *poznaniensis* Zaleski	P. 69	19	0·2	36·3
		56	0·0	55·0
P. Niklewskii Zaleski	P. 61	26	0·9	36·2
		65	0·1	37·6

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Table 1 (*cont.*).

Organism	Catalogue no.*	Incubation period (days)	Residual glucose by polarimeter (g./100 ml.)	Utilization of SO_4 (%)
P. obscurum Biourge	P. 87	21	1·6	35·8
		68	0·0	50·7
P. fellutanum Biourge	P. 60	26	2·1	35·4
		65	0·1	50·2
P. Biourgei Dierckx	P. 73	20	1·8	35·0
		67	0·0	39·8
P. roseo-citreum Biourge	P. 94	25	1·4	34·1
		69	0·9	37·2
P. Adametzi Zaleski	P. 58	25	2·5	34·0
		64	0·1	58·2
P. granulatum Bainier	P. 35	22	0·4	32·8
		38	0·2	45·4
P. flavi-dorsum Biourge	P. 79	20	2·2	32·4
		70	0·0	43·2
P. gorgonzola Weidemann	P. 83	21	2·6	32·3
		69	0·1	53·3
P. subcinereum Westling	P. 98	25	2·8	32·0
		69	0·1	38·4
P. terlikowskii Zaleski	P. 63	18	2·5	30·1
		67	0·2	54·2
P. decumbens Thom	P. 76	20	2·9	29·4
		71	0·0	40·6
P. chrysogenum Thom	P. 18	21	2·4	29·2
		60	0·1	53·3
P. brunneo-rubrum Dierckx	P. 9	18	2·9	28·8
		63	0·8	50·1
P. frequentans Westling	P. 80	20	3·2	28·8
		70	0·0	44·2
P. chrysogenum Thom	P. 17	21	3·3	27·6
		61	1·5	34·4
P. frequentans Westling	P. 82	21	3·1	27·1
		70	0·1	65·4
P. carmino-violaceum Dierckx	P. 64	18	3·3	26·7
		71	0·0	50·7
P. baiiolum Biourge	P. 70	19	1·4	25·8
		67	0·0	43·2
P. Paczoskii Zaleski	P. 62	18	2·5	25·0
		71	0·0	45·0
P. aurantio-virens Biourge	P. 4	20	3·5	22·9
		107	0·0	32·0
P. meleagrinum Biourge	P. 43	23	3·4	22·7
		78	2·2	36·7
P. atramentosum Thom	P. 1	20	3·4	21·7
		64	0·1	47·7
P. griseo-fulvum Dierckx	P. 68	19	3·5	20·1
		71	0·0	74·8
P. flavo-cinereum Biourge	P. 77	20	3·5	19·6
		70	0·1	44·5
P. spiculisporum Lehman	P. 53	25	1·6	17·0
		43	0·1	20·0
P. schneggii Boas	P. 51	24	4·2	12·4
		64	2·4	32·8
P. minio-luteum Dierckx	P. 44	23	3·8	11·7
		78	1·8	28·0
P. jantho-citrinum Biourge	P. 40	23	3·8	10·5
		78	3·2	25·8
P. aurantio-candidum Dierckx	P. 3	20	4·5	7·0
		65	3·6	42·6
P. brunneo-violaceum Biourge	P. 10	18	2·8	5·8
		61	0·2	73·4

* London School of Hygiene and Tropical Medicine (L.S.H.T.M.)

Table 1 (*cont.*).

Organism	Catalogue no.*	Incubation period (days)	Residual glucose by polarimeter (g./100 ml.)	Utilization of SO_4 (%)
	Species of *Aspergillus*			
A. fumigatus Fresenius	No. 47	19	2·4	99·3
		47	0·3	85·1
A. terreus Thom	Am. 1	17	0·5	93·0
		59	0·0	82·5
A. terreus Thom	No. 3	17	0·6	90·5
		59	0·0	98·2
A. terreus Thom	Ab. 2	19	0·4	85·5
		57	0·0	97·2
A. terreus Thom	No. 37	17	0·8	75·6
		59	0·0	99·5
A. flavus Link.	No. 51	19	0·5	74·8
		47	0·2	98·7
A. terreus Thom	Ac. 24	17	0·5	71·1
		57	0·6	100·0
A. Sydowi Bainier & Sartory	No. 43	18	0·6	68·5
		46	0·0	88·5
A. terreus Thom	Am. 2	19	0·2	67·1
		57	0·0	97·7
A. ochraceous Wilhelm	No. 69	19	1·5	66·2
		47	0·2	97·9
A. terreus Thom	Ab. 1	19	0·8	61·0
		59	0·0	69·9
A. terreus Thom	Ac. 107	17	0·3	58·5
		57	0·0	63·9
A. oryzae (Ahlburg) Cohn	A. 11	25	0·5	57·0
		48	0·3	50·7
A. Wentii Wehmer	No. 2	19	2·0	42·8
		46	0·6	37·1
A. nidulans Eidam	No. 46	18	1·6	41·4
		46	0·1	69·0
A. versicolor Vuill. (Tiraboschi)	No. 18	19	2·2	41·0
		46	0·1	62·2
A. terreus Thom	Ac. 100	17	2·6	31·6
		60	0·0	57·9
A. tamarii Kita	No. 55	20	3·4	19·7
		47	0·5	42·8
A. candidus Link.	No. 32	25	3·8	17·9
		49	0·1	44·5
	Miscellaneous species			
Clasterosporium sp.	Ag. 64	18	0·7	39·7
		46	0·1	42·0
Stysanus stemonites (Pers.) Corda	No. 137	20	2·4	33·2
		48	0·1	41·0
Stemphylium lanuginosum Harz.	No. 56	20	4·2	31·5
		47	2·5	21·8
Syncephalastrum cinereum Bainier	No. 112	20	4·4	22·7
		48	0·4	36·2
Absidia spinosa Lendner	No. 118	20	4·0	19·7
		48	0·6	29·7
Alternaria sp.	Ag. 17	20	4·3	11·8
		49	3·3	24·0
Trichoderma viride Pers. ex Fries	No. 85	19	4·1	11·3
		48	1·8	24·0
Caldariomyces fumago Woronichin	Ag. 92	18	2·9	7·0
		46	1·5	68·4
Cephalosporium sp.	Ag. 30	25	4·3	4·8
		49	3·1	14·4

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the amount of sulphate metabolized after the shorter incubation period. Eighty-seven species or strains of *Penicillium*, 19 of *Aspergillus* and 9 miscellaneous species were examined.

Sulphur balance sheets of fourteen species or strains of moulds

The aims of this part of the investigation which, because of the large number of estimations involved, was of necessity somewhat restricted, were twofold:

(*a*) To explore the variations in sulphur metabolism of different strains within a single species of mould. For this purpose six authentic strains of *Aspergillus terreus* Thom were chosen, since it was already known, from investigations carried out in this laboratory, that different strains of this species give rise to a large number of different metabolic products. Two of these strains, no. 45 and Am. 2, metabolize considerable amounts of inorganic chloride (Clutterbuck *et al.* 1940).

(*b*) To explore the variations in sulphur metabolism of different species of moulds within a single genus. Seven different species of *Penicillium* were chosen for this purpose.

Eight flasks, each containing 350 ml. of Czapek-Dox medium, were sterilized, inoculated with the species or strain under investigation, and incubated at 24°. Four flasks of each strain or species were harvested, and the filtered contents made up to 2 l. after (*a*) substantially all the glucose had been utilized, and four flasks after (*b*) extended incubation for 9–11 weeks. In the culture filtrate, residual inorganic sulphate, sulphate generated after acid hydrolysis and total sulphur in solution were determined, and total sulphur in the mycelium, by the methods previously described. The results obtained are given in Table 2.

DISCUSSION

An outstanding fact, that emerges from the results presented in Table 1, is the evident importance of sulphur in the metabolism of moulds, as indicated by the large amounts of inorganic sulphate absorbed from the nutrient medium. Thus, of the 115 species or strains examined, seven species of *Penicillium* and nine species or strains of *Aspergillus*, including six strains of *A. terreus* Thom, utilized, after either the shorter or longer incubation period, 90·0 % or more of the inorganic sulphate originally present in the nutrient medium. Similarly, 40 species or strains utilized from 60·0 to 89·9 % of the inorganic sulphate. With 29 species or strains the percentage utilization of inorganic sulphate was more than 5 % greater after the shorter period of incubation than after the longer period. In some of these cases, where the difference is relatively small, it is possible that the difference represents variations between single flasks. In others, it is probably due to the hydrolysis of 'ethereal sulphates' formed as metabolic products (see below).

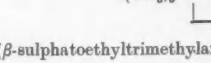
The seven different species of *Penicillium*, six different strains of *Aspergillus terreus* Thom and one of *A. fumigatus* Fresenius, showing a large percentage utilization of inorganic sulphate, were chosen for a more detailed examination of their sulphur metabolism with the results given in Table 2. Consideration of these results leads to the following conclusions:

(1) *Utilization of inorganic sulphate* (column 6, Table 2). All seven species or strains of *Aspergillus* and three species of *Penicillium* utilized the whole of the inorganic sulphate originally present in the medium within the limits of experimental error ± 5 %, and after either the shorter or longer period of incubation. Two of the four remaining species utilized over 90 %. The results are thus in substantial agreement with those given in Table 1.

(2) *Inorganic sulphate generated after acid hydrolysis* (column 8, Table 2). With most species or strains examined the amount of inorganic sulphate regenerated after acid hydrolysis under relatively mild conditions (boiling with 0·3 N-HCl for 30 min.) is considerable, reaching a maximum of 60·1 % with *Penicillium citreo-roseum*, and exceeding 25 % with ten other species or strains. The nature of this sulphur has not yet been established, but it is clear that it is present in some relatively labile form possibly as 'ethereal sulphates'.

The presumed formation of 'ethereal sulphates' does not, however, appear to be a property possessed by all the species examined. Thus the percentage amount of 'ethereal sulphates' formed by *P. notatum* (5·3 and 3·9 % after 35 and 75 days' incubation respectively), and by *P. aurantio-brunneum* (5·2 % after 36 days' incubation) falls within, or very close to, the experimental error of the method of estimation (± 5 %). A similar observation was made by Rippel & Behr (1936) who cultivated *Aspergillus niger* on a chemically defined medium, somewhat different in nature to Czapek-Dox medium, and containing the sulphates of potassium, magnesium and zinc. They observed the utilization of inorganic sulphate, and showed that boiling the metabolism solution with 0·5 N- or N-HCl for 2 hr. gave no appreciable increase in inorganic sulphate. We have confirmed the absence of 'ethereal sulphate' formation by our strain of *A. niger* L.S.H.T.M. strain Ac. 1 when grown on Rippel & Behr's medium.

So far as we are aware only one 'ethereal sulphate' has been isolated in a state of purity from mould cultures. Woolley & Peterson (1937) extracted cyclic choline sulphate



(β-sulphatoethyltrimethylammonium betaine

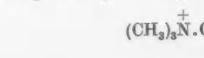


Table 2. *Balance sheet showing distribution of sulphur originally supplied as inorganic sulphate and metabolized by fourteen species or strains of moulds*

Organism	Catalogue no.*	Incubation period (days)	Residual glucose (g./100 ml.)	Weight of mycelium (g./flask)	Residual inorganic SO_4 I (%)	Total SO_4 after hydrolysis II (%)	SO_4 generated after hydrolysis III (%)	Total S in filtrate IV (%)	Non-SO_4-S after hydrolysis V (%)	S in mycelium VI (%)	Total S accounted for IV + VI (%)
A. terreus	Ac. 24	29	0·2	3·01	0·0	26·8	26·8	66·9	40·1	32·2	99·1
		71	—	2·18	0·5	16·2	15·7	70·8	54·6	21·8	92·6
A. terreus	No. 45	29	0·2	3·19	0·0	19·8	19·8	52·8	33·0	47·2	100·0
		78	—	1·92	0·0	35·5	35·5	74·7	39·2	29·4	104·1
A. terreus	No. 3	22	0·1	5·78	2·9	18·5	15·6	37·4	18·9	52·6	90·0
		71	—	3·41	0·5	27·1	26·6	48·5	21·4	40·0	88·5
A. terreus	Ab. 2	33	0·1	5·45	12·0	35·5	23·5	49·4	13·9	46·3	95·6
		62	—	4·56	0·0	43·5	43·5	53·8	10·3	47·6	101·4
A. terreus	No. 37	30	0·1	6·04	14·0	27·2	13·2	41·0	13·8	56·8	97·8
		63	—	4·73	0·0	39·0	39·0	51·5	12·5	44·6	96·1
A. terreus	Am. 2	30	0·1	3·40	19·0	35·4	16·4	53·7	18·3	40·3	94·0
		74	—	2·57	1·2	27·8	26·6	49·8	22·0	37·6	87·4
A. fumigatus	No. 47	41	1·5	3·00	0·0	16·5	16·5	48·1	31·6	37·9	86·0
		76	—	3·35	0·7	16·5	15·8	43·2	26·7	50·3	93·5
P. citreo-nigrum	P. 20	41	0·0	2·49	0·0	40·6	40·6	59·9	19·3	32·7	92·6
		74	—	2·15	3·5	45·0	41·5	70·0	25·0	24·0	94·0
P. olivino-viride	P. 46	35	0·0	2·46	6·6	52·1	45·5	83·9	31·8	15·9	99·8
		70	—	1·96	6·5	49·4	42·9	77·8	28·4	22·6	100·4
P. puberulum	P. 47	27	0·0	2·79	4·9	36·1	31·2	64·6	28·5	28·5	93·1
		70	—	1·79	0·0	20·6	20·6	68·1	47·5	27·2	95·3
P. expansum	P. 41	41	0·4	2·13	34·0	63·1	29·1	75·6	12·5	23·1	98·7
		68	—	1·97	28·2	62·5	34·3	77·4	14·9	26·4	103·8
P. citreo-roseum	P. 22	36	0·2	3·65	21·8	43·6	21·8	59·0	15·4	40·6	99·6
		67	—	3·30	4·5	64·6	60·1	72·1	7·5	31·4	103·5
P. notatum	P. 45	35	0·0	2·26	0·4	5·7	5·3	70·0	64·3	24·8	94·8
		75	—	1·75	8·4	12·3	3·9	96·6	84·3	7·4	104·0
P. aurantio-brunneum	P. 2	36	0·0	3·98	23·4	28·6	5·2	45·9	17·3	43·3	89·2
		68	—	2·80	17·4	26·8	9·4	49·8	23·0	50·6	100·4

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as colourless crystals from the mycelium of *A. Sydowi* Bainier & Sartory grown on a glucose-mineral salts medium. This substance yields choline and sulphuric acid on acid hydrolysis.

(3) *Sulphur in the culture filtrate not present as inorganic sulphate after acid hydrolysis* (column 10, Table 2). All the species and strains examined convert considerable amounts of inorganic sulphate into water-soluble, sulphur-containing compounds, which do not give rise to inorganic sulphates on mild acid hydrolysis. In some cases the conversion is surprisingly large, e.g. *Penicillium notatum*, 75 days' incubation, 84·3 %; *Aspergillus terreus*, strain Ac. 24, 71 days' incubation, 54·6 %; *Penicillium puberulum*, 70 days' incubation, 47·5 %.

Nothing is known at present as to the nature of these sulphur compounds, though it is probable that the sulphur is in organic combination. Hydrogen sulphide and mercaptans were not encountered, although Birkinshaw, Findlay & Webb (1942) established the formation of methyl mercaptan by the wood-rotting fungus *Schizophyllum commune* Fr., when grown on a synthetic medium containing glucose with ammonium and magnesium sulphates as sources of sulphur.

It is improbable that the sulphur is present to any considerable extent as protein or peptone. Thus, although Clutterbuck, Lovell & Raistrick (1932) isolated from culture filtrates of strains of *Penicillium chrysogenum* Thom and *P. notatum* Westling, including the strain P. 45, a protein containing 3·5 % of cystine, the yield, expressed as a percentage of the inorganic sulphate in the medium, is small. Similarly, Rippel & Behr (1936) state that, of the inorganic sulphate utilized by *Aspergillus niger*, less than one seventh is present as protein and none as peptone.

Very few mould metabolic products containing sulphur have been isolated in a state of purity. It may be significant, however, that the different penicillins, metabolic products of *Penicillium notatum*, all give $\beta\beta$-dimethylcysteine on hydrolysis, and that another antibiotic, gliotoxin, produced by a variety of fungi, including *Aspergillus fumigatus* Fresenius (Menzel, Wintersteiner & Hoogerheide, 1944) and *Penicillium cinerascens* Biourge (Bracken & Raistrick, 1947), has the empirical formula $C_{13}H_{14}O_4N_2S_2$.

Ovcharov (1937) has demonstrated qualitatively the formation of thiourea by cultures of *Verticillium albo-atrum* and *Botrytis cinerea* grown on Richards's (1897) medium, containing magnesium and ferrous sulphates as sole source of sulphur.

(4) *Sulphur in mycelium* (column 11, Table 2). Although the total sulphur in the mycelium amounts in some cases, particularly with strains of *Aspergillus terreus*, to about half that supplied originally as inorganic sulphate, the percentage of sulphur in the dried mycelium is, with three exceptions, fairly uniform, and is consistently small. The percentage sulphur content in the dried mycelium of the different species and strains examined is as follows (*d.* = incubation period in days): *A. terreus*, Ac. 24. 29*d.*, 0·24 %; 71*d.*, 0·23 %. *A. terreus*, no. 45. 29*d.*, 0·34 %; 78*d.*, 0·35 %. *A. terreus*, no. 3. 22*d.*, 0·21 %; 71*d.*, 0·27 %. *A. terreus*, Ab. 2. 33*d.*, 0·20 %; 62*d.*, 0·24 %. *A. terreus*, no. 37. 30*d.*, 0·22 %; 63*d.*, 0·22 %. *A. terreus*, Am. 2. 30*d.*, 0·27 %; 74*d.*, 0·33 %. *A. fumigatus*, no. 47. 41*d.*, 0·29 %; 76*d.*, 0·34 %. *Penicillium citreo-nigrum*, P. 20. 41*d.*, 0·30 %; 74*d.*, 0·25 %. *P. olivino-viride*, P. 46. 35*d.*, 0·15 %; 70*d.*, 0·26 %. *P. puberulum*, P. 47. 27*d.*, 0·23 %; 70*d.*, 0·35 %. *P. expansum*, P. 41. 41*d.*, 0·25 %; 68*d.*, 0·31 %. *P. citreo-roseum*, P. 22. 36*d.*, 0·25 %; 67*d.*, 0·22 %. *P. notatum*, P. 45. 35*d.*, 0·25 %; 75*d.*, 0·10 %. *P. aurantio-brunneum*, P. 2. 36*d.*, 0·25 %; 68*d.*, 0·41 %.

Thus, although the maximum and minimum weights of dried mycelium/flask for the shorter incubation period are 6·04 g. (*Aspergillus terreus*, no. 37, 30 days) and 2·13 g. (*Penicillium expansum*, P. 41, 41 days), their respective sulphur contents are 0·22 and 0·25 %.

The dry weight of mycelium (Table 2, column 5) is less after the longer period of incubation than after the shorter period, in every case except for *Aspergillus fumigatus*, no. 47, clearly indicating that autolysis has taken place in the interval. Disregarding any results which fall within the limits of experimental error (± 5 %) there has been during this interval:

(*a*) A loss of sulphur from the mycelium to the metabolism solution in seven cases (limits − 8·7 % with *Penicillium citreo-nigrum*, P. 20, to − 17·8 % with *Aspergillus terreus*, no. 45) and a gain in three cases (limits + 6·7 % with *Penicillium olivino-viride*, P. 46, to + 12·4 % with *Aspergillus fumigatus*, no. 47).

(*b*) A gain in 'ethereal sulphates' in the metabolism solution in seven cases (limits + 5·2 % with *Penicillium expansum*, P. 41, to + 38·3 % with *P. citreo-roseum*, P. 22) and a loss in two cases (− 10·6 % with *P. puberulum*, P. 47, and − 11·1 % with *Aspergillus terreus*, Ac. 24).

(*c*) A gain in sulphur compounds, other than 'ethereal sulphates', in the metabolism solution in six cases (limits + 5·7 % with *Penicillium citreo-nigrum*, P. 20, and *P. aurantio-brunneum*, P. 2, to + 20·0 % with *P. notatum*, P. 45) and a loss in one case of − 7·9 % with *P. citreo-roseum*, P. 22.

SUMMARY

1. A survey has been made of the utilization of inorganic sulphate by 115 species or strains of moulds, grown on Czapek-Dox glucose solution con-

taining magnesium and ferrous sulphates as the sole source of sulphur.

2. This has demonstrated the universal importance of inorganic sulphates in the metabolism of moulds, since all species or strains tested utilized 10% or more, 56 utilized 60% or more, and 16 over 90% of the inorganic sulphate in the medium.

3. A more detailed examination of the sulphur metabolism of seven species of *Penicillium*, six strains of *Aspergillus terreus* Thom, and one strain of *A. fumigatus* Fresenius, grown on the same medium, revealed the fact that some of them form considerable amounts of 'ethereal sulphates' while others, particularly *Penicillium notatum* Westling, give large yields of other, probably organic, sulphur compounds, of at present unknown nature.

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Studies on Ruminant Saliva

I. THE COMPOSITION AND OUTPUT OF SHEEP'S SALIVA

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In all domestic animals the principal function of saliva is that of a lubricant to assist mastication and deglutition. In ruminants however, saliva has a still more important role, for the rumen has no secretion of its own and the saliva forms a fluid medium for the transport of ingesta both back to the mouth for remastication and onwards through the stomach to the small intestine. It forms also a buffered medium in which the micro-organisms of the rumen can flourish. Particular interest therefore is attached to the saliva of the ruminant, to its composition and the amounts in which it is formed.

The literature on ruminant saliva is not very extensive: except for a few more recent papers, most of it has been reviewed by Mangold (1929). The sublingual and submaxillary glands, when stimulated by eating, secrete a saliva of low ash and alkalinity which contains mucin and whose function is limited to lubricating the bolus of food. The parotid gland, on the other hand, secretes during eating and rumination, at a greater rate on the side on which the bolus is being masticated, and its flow continues at a reduced rate during the intervals of rest. The parotid saliva is formed in large amounts, it contains no ptyalin but has a high alkalinity and buffering power. It is consequently the most important secretion for the maintenance of normal rumen function.

The output of saliva by various ruminants as reported in the literature is summarized in Table 1. The quantity produced per day has been estimated at 56 kg. for the ox by Colin (1886) and at 50 l. for the

Table 1. *Published values for outputs of ruminant saliva*

Species	Secretion	Output of saliva ml./hr.	Output of saliva Total/day	Reference
Ox	Parotid	800–2400	56 kg.	Colin (1886)
Cow	Mixed	—	50 l.	Markoff (1913)
Sheep	Parotid	25	—	Scheunert & Trautmann (1921)
	Parotid	3·6–21·6	—	Popov (1921)*
Goat	Parotid	10	—	Do.
	Parotid	60	—	Babichev (1925)*

* Quoted by Babkin (1944).

Table 2. *Published composition of ruminant saliva**

Animal	Secretion	Content (g./100 ml.)			Content (mg./100 ml.)						CO_2 content (ml./100 ml.)	pH	Reference
		Dry matter	Ash	Alkalinity as soda	N	Na	K	Ca	Mg	P	Cl			
Sheep	Parotid	1·1 –1·25	0·75–0·9	0·56–0·77									Scheunert & Trautmann (1921)
				0·56–0·77									Schwarz & Rasp (1926)
												8·09–8·42	Popov (1932)†
	Mixed		1·2			363	29			52	22		Scheunert & Krzywanek (1930)
										20–60			Watson (1933)
												8·23–8·54	Weyers (1937)
	Rumen liquor	1·8	0·94					26		87	50		Brumnich & Winks (1931)
Goat	Parotid	0·71–1·96	0·02–1·02		10–46						10–12		Trautmann & Albrecht (1931)
												8·12–8·32	Popov (1932)†
Ox	Mixed	1·25–1·67	0·83–0·86	0·6									Markoff (1913)
Cattle	—											8·1	Schwarz & Hermann (1924)
	Mixed	0·42–2·2										8·8	Chrzaszcz & Schechlowna (1930)
Buffalo	Parotid	0·85	0·68		Trace	277		6	359	15		8·8	Sharma (1936)

* To facilitate comparison, some data have been expressed in a form different from the original.

† Quoted by Babkin (1944).

cow by Markoff (1913), the diet being hay in each case. The analyses of the saliva of various ruminants reported in the literature are given in Table 2. Zuntz (1913) claimed that the amount of alkali secreted each day in the saliva of the ox must be about six times that contained in the blood and Markoff (1913) calculated that his cow produced 300–350 g. alkali expressed as sodium bicarbonate. The latter figure may be compared with the value of 329 g. volatile acid (calculated as acetic acid) in the rumen and reticulum of an ox, reported by Elsden, Hitchcock, Marshall & Phillipson (1946). It is evident that in this species the saliva flowing into the rumen each day supplies a large volume of liquid for the suspension of the ingesta and a large amount of alkali for the neutralization of acids produced in the rumen.

The analyses in Table 2 are rather incomplete, often being limited to the dry matter and titratable alkalinity. In view of the importance of the saliva to the ruminant, a more detailed knowledge of its composition seemed to be required.

In the present work a study has been made of both the mixed and parotid secretions of sheep. First, the composition was investigated by analyzing samples for dry matter, ash and various constituents, particularly inorganic ions, which might be supplied from the blood of the animal. Secondly, special attention was paid to the determination of the carbon dioxide content and the hydrogen-ion concentration. During the investigations a number of observations was also made on the rate of secretion and the daily output of saliva.

METHODS

Collection of samples. Mixed saliva was obtained either as it dripped from the tongue of a sheep under chloralose or Nembutal (pentobarbital) anaesthesia, or from a normal sheep by means of a sponge gag, Pl. 1 *a* and *b*, devised for the purpose. The samples were usually uncontaminated, though occasionally they appeared to contain small amounts of regurgitated rumen contents.

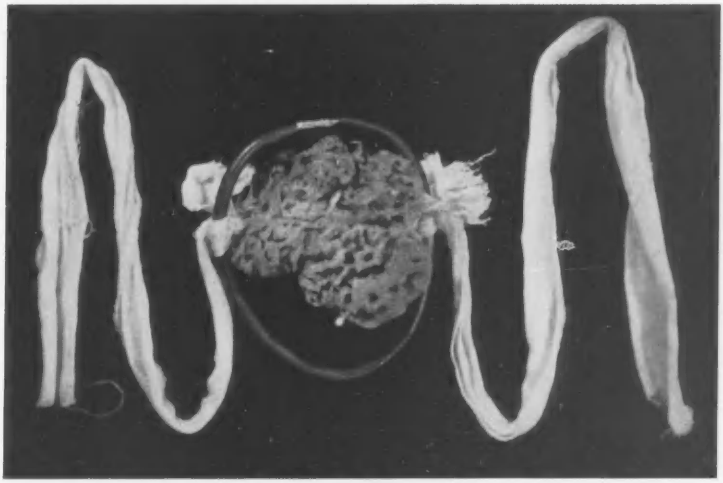
Parotid saliva was obtained by cannulating the duct from the parotid gland under Nembutal anaesthesia and leading the saliva off through valve-rubber tubing, Pl. 1 *c*. Collection of samples was commenced about 1 hr. after the effect of the anaesthetic had ceased and a portion was taken under paraffin oil to prevent loss of carbon dioxide for the estimation of the pH and carbon dioxide content.

Rumen liquor was obtained either from a sheep with a rumen fistula or from freshly killed animals. Care was taken to avoid undue exposure to air by covering with a layer of liquid paraffin, and bacterial activity was arrested by the addition of merthiolate (sodium ethylmercurithiosalicylate).

Chemical methods. The dry matter and ash were determined by pipetting 5 ml. of saliva into a small tared crucible, evaporating on a water bath and drying in a hot-air oven at 110° to constant weight; finally it was ashed and weighed again.

(*a*) Mixed saliva.

(*c*) Parotid saliva



(*b*) Sponge gag.

E. I. McDOUGALL—STUDIES ON RUMINANT SALIVA

Nitrogen was determined by the micro-Kjeldahl method, reducing substances by Maclean's method for blood sugar, the carbon dioxide content with Van Slyke's volumetric apparatus, and the hydrogen-ion concentration potentiometrically using a quinhydrone electrode.

Inorganic ions were estimated by standard methods for blood serum as follows: sodium, Kramer & Gittleman (1924); potassium, Kramer & Tisdall (1921); calcium, Clark & Collip (1925); magnesium, Denis (1922); phosphorus, Fiske & Subbarow (1925); chloride, at first by the method of Whitehorn (1921) and later by the method of Sendroy (1937).

RESULTS

Detailed analyses of saliva

Mixed saliva. Eleven samples were obtained from four anaesthetized and one normal sheep. The particulars of the samples and the results of the analyses are given in Table 3.

The dry matter was 1·0–1·39 and the ash 0·73–0·9 g./100 ml.; the organic matter was thus quite low. A small amount of nitrogen was found but its nature was not determined. No reducing sugars were detected. The chief constituents were inorganic: sodium and bicarbonate predominated; potassium, chloride and phosphate were present in smaller amounts, and calcium and magnesium were found in traces.

As the samples were not very uniform in origin they call for individual comment. With sheep 2, sample A was obtained early, and B late, in the period of anaesthesia; the analyses show, however, that a fairly steady composition was being maintained. Of the samples from sheep 3 and 4, sample *a* appeared to contain a small amount of regurgitated rumen contents, whereas sample *b* was uncontaminated; it is evident that the effect of this contamination was negligible.

Four samples were obtained from sheep 5 at intervals during 24 hr. by means of the sponge gag. Some constituents, notably the phosphorus and carbon dioxide, showed considerable variation during this period.

The differences in the composition of saliva from different animals were more marked for certain constituents, particularly potassium, phosphorus and carbon dioxide, than for others. These differences, however, cannot be understood without an adequate knowledge of the composition of the individual salivary secretions. Of the latter, that from the parotid gland is the most important and has therefore received special attention in this investigation.

Parotid saliva. Samples were obtained at intervals throughout the day from six sheep with parotid fistulae. Of these sheep, no. 2 and three others were wethers, no. 4 an approximately 6 months old lamb and one an aged ram. For four of the animals the experiments lasted until the third day after cannulation by which time the rate of production of saliva had become inconveniently slow. In two animals the experiments were stopped on the 1st and 2nd days after cannulation respectively as the secretion had dried up and become almost negligible. At the end of the experiment one sheep had an infection of the duct but not the reduction in size and structural alteration in the gland reported by Scheunert & Trautmann (1921) to occur some time after establishment of a parotid fistula. In two other animals examined there was no apparent reason for the stoppage of secretion; in fact, the gland subsequently healed up and was observed to function normally again.

Of the results obtained, those for two sheep only are given in Table 4 because of limitations of space. In addition to these analyses, a number of samples was examined qualitatively by the biuret, ferric chloride and Benedict tests for protein, thiocyanate and reducing sugars but all these tests proved negative.

The results for sheep 2 showed lower values for carbon dioxide content and pH than those for the other sheep and are considered atypical though the reason for their being so is not apparent. The results for sheep 4 were typical of those obtained for the four remaining sheep. Only those samples collected on the first 2 days of the experiments on these five animals have been considered as normal. Those obtained on the 3rd day were excluded in taking average values because by this time the secretion was drying up as already noted and also changes in its composition had often become evident. On the whole these results showed that the composition of the parotid saliva was not affected by the age of the animals, that it was similar to that of the mixed saliva quoted in Table 3, and hence that the latter secretion was largely composed of parotid saliva.

The results for the cations do not call for much comment but those for the anions showed the following features. The phosphorus (inorganic), average 81 mg./100 ml., showed extensive variations, both between one animal and another and among samples from the same animal. The values tended to decrease slightly when the animal was eating or ruminating and to fall gradually during the course of the experiment to a fraction of the original amount. The chloride content, average 61 mg./100 ml., also varied definitely from animal to animal but, on the other hand, it increased considerably during the course of the experiment; in the most extreme case, that of sheep 4, from 23 to 238 mg./100 ml. These two effects were approximately compensatory, as the third chief anion, the carbon dioxide content, was more steady, though in three cases there were indications of lower values setting in on the 3rd day of an experiment. Its value, average 233 ml./100 ml., was approximately equivalent to 0·1 N-bicarbonate solution and showed an

Table 3. *Composition of the mixed saliva of sheep*

Sheep no.	Sample	Content (g./100 ml.) Dry matter	Ash	Reducing compounds	Content (mg./100 ml.) N	Na	K	Ca	Mg	P (inorg.)	Cl	CO_2 (ml./100 ml.)	pH
					(*a*) Anaesthetized sheep								
1	—	1·0	0·8	—	—	428	39	3·0	—	37	25	283	8·6
2	A	—	—	0	—	370	16	1·6	0·8	57	35	207	8·6
	B	—	—	0	9	392	22	2·0	0·7	52	41	212	8·5
3	*a*	1·2	0·9	0	—	399	29	2·3	1·0	72	34	221	8·4
	b	—	—	—	19	414	30	—	0·9	83	—	200	—
4	*a*	1·0	0·9	0	—	399	26	2·5	0·8	38	43	255	8·6
	b	1·0	0·9	0	20	387	—	2·4	0·6	—	43	252	—
					(*b*) Normal sheep								
5	5 p.m.	—	—	—	—	429	45	2·3	—	52	—	176	8·6
	9.30 a.m.*	1·10	0·73	—	—	429	45	2·3	—	53	—	117	8·7
	12.30 p.m.	1·39	0·90	—	—	462	46	2·1	—	86	—	152	8·6
	4.30 p.m.	1·39	0·89	—	—	462	40	2·4	—	62	—	172	8·5

Treatment: sheep 1, chloralose anaesthesia; sheep 2–4, Nembutal. Samples A and B collected early and late in period, under anaesthetic. Sample *a*: slight contamination suspected with regurgitated rumen contents; sample *b*: no contamination evident.
* Before feeding.

Table 4. *Composition of the parotid saliva of sheep*

Sheep no.	Sample: Day of exp.	Sample: Time	Content (g./100 ml.) Dry matter	Ash	Content (mg./100 ml.) N	Na	K	Ca	Mg	P	Cl	CO_2 (ml./100 ml.)	pH	Time to secrete 10 ml. samples (*a*) min.	(*a*) sec.	(*b*) min.	(*b*) sec.	(*c*) min.	(*c*) sec.
2	1	12.30 p.m.	1·38	1·15	20	441	39	1·5	1·0	106	77	106	7·69	—		—		—	
		4.30 p.m.	—	—	—	447	45	1·3	1·1	120	—	118	7·79	—		—		—	
		7.30 p.m.	1·28	1·10	16	425	35	2·0	1·0	129	89	146	—	—		—		—	
4	1	12 noon	1·30	1·00	17	410	12	0·4	0·5	92	23	246	8·18	10	0	9	0	7	30
		1 p.m.*	1·25	0·95	21	396	14	1·0	1·3	64	19	267	8·20	2	0	3	0	5	0
		4.30 p.m.	1·30	1·05	23	424	19	0·3	0·5	102	19	266	8·23	6	0	7	45	—	
		11 p.m.	1·35	1·15	24	439	18	0·2	0·5	125	29	249	8·17	10	30	8	30	—	
	2	5.30 a.m.	1·30	1·10	28	412	22	0·7	0·7	82	51	285	8·06	8	0	7	0	—	
		10 a.m.	1·20	0·90	25	394	19	0·7	0·5	59	80	245	8·20	6	30	7	30	5	30
		10.30 a.m.*	1·15	0·85	23	387	25	0·6	0·4	48	94	250	8·23	2	45	3	30	3	40
		5 p.m.	—	—	31	391	22	0·1	0·8	57	88	207	8·04	13	0	21	0	30	0
		11 p.m.	1·25	0·90	35	395	22	0·5	0·9	79	99	202	8·03	30	0	26	0	—	
	3	6 a.m.†	1·10	0·80	29	352	26	0·6	1·1	28	174	205	8·15	4	0	4	30	—	
		10 a.m.	—	—	31	356	15	0·6	0·9	28	148	201	8·12	18	0	27	30	—	
		11 a.m.*	1·10	0·90	25	357	14	0·6	0·5	19	180	191	8·08	12	0	7	0	—	
		3.40 p.m.‡	—	—	29	370	13	0·8	0·5	20	238	172	7·97	—		10	30	—	

Sheep 1, 3, 5 and 6 gave similar results to sheep 4.
* Feeding. † Ruminating. ‡ After injection of pilocarpine.

increase during eating, when the rate of secretion was also higher.

The form of phosphorus in saliva. In the analyses recorded above it was the inorganic phosphorus that was determined. To see to what extent this accounted for all the phosphorus and whether other forms were secreted from the blood, both total and inorganic phosphorus were determined in a number of samples of mixed and parotid saliva. The results indicated that, within the limits of experimental error, all of the phosphorus exists in the inorganic form.

The carbon dioxide content and hydrogen-ion concentration of saliva

The carbon dioxide content of mixed saliva. This quantity is important as it is the best indicator of the buffering power of the liquid flowing into the rumen. As it appeared from the analyses of the mixed saliva of sheep 5, Table 3, that this constituent may vary considerably throughout the day, it was decided to investigate it further. For this purpose samples of mixed saliva were collected at regular intervals during the day, from seven normal sheep, by means of the sponge gag.

At first the carbon dioxide content was determined on the samples as collected, but as the mixed secretion cannot be obtained without exposure to the air, the question arose whether the carbon dioxide content, and consequently the hydrogen-ion concentration of samples collected in this way, were representative of the original secretion. It was possible to investigate this point when studying the parotid secretion (see later). Samples were collected under liquid paraffin from five sheep from a cannula in the parotid duct. The carbon dioxide content was determined on the sample as collected. The sample was then exposed to the air overnight and the carbon dioxide content redetermined. The exposed sample was then equilibrated with human alveolar air and the carbon dioxide content measured again. The three sets of figures showed that there was usually a loss of carbon dioxide on exposure of the sample to the air, but that the initial values were restored by equilibration with human alveolar air, although no data could be found in the literature to compare the partial pressure of the carbon dioxide in the alveolar air of the sheep with that in man. The remaining samples of mixed saliva were therefore analyzed after equilibration to obtain the true value of the carbon dioxide content. Some samples were analyzed both before and after equilibration in order to determine the magnitude of the error involved in collecting the sample.

The results are given in Table 5. The unequilibrated samples *a* showed the same general trends as the equilibrated samples *b* but were some 10–20 % lower. Therefore the figures given in

Table 5. CO_2 *content of the mixed saliva of sheep*

(*a* = untreated sample. *b* = sample equilibrated with alveolar air.)

Sheep no. and treatment ...	1*a*	2*a*	3*a*	4*a*	4*a*	3*a*	3*b*	5*a*	5*b*	6*a*	6*b*	1*b*	2*b*	7*b*
	CO_2 (ml./100 ml.)													
Time														
9.30 a.m.	31	70	127	156	131	112	135	85	105	135	157	52	43	114
12.30 p.m.	40	54	78	64	48	84	106	171	200	147	181	38	90	92
3.30 p.m.	—	—	—	—	—	—	—	81	117	117	139	49	92	107
5.30 p.m.	60	164	84	123	61	143	153	67	81	79	91	25	45	109
9.30 p.m.	42	56	61	88	69	173	183	77	87	81	95	36	113	143

Table 3, and probably most of those hitherto reported in the literature for ruminant saliva, must be regarded as indicating too low a carbon dioxide content and thus too alkaline a reaction.

The variation in the carbon dioxide content at different times of the day was often as great as 100% but was not regular enough to suggest any correlation with the time of feeding which took place after the first sample had been taken. Such a correlation might become apparent if the observations were repeated with more detailed information available about the feeding and ruminating habits of the animals. As the variation was greater than that reported in Table 4 for parotid saliva, it was probably due to changes in the proportions, rather than the composition, of the individual secretions.

The carbon dioxide content of these samples was on the whole lower than was recorded for the anaesthetized sheep in Table 3; the values after equilibration showed a range of 25–200 ml./100 ml. The lowest figures were mainly due to sheep 1 and 2,* but it is not possible to say that these were definitely abnormal owing to the small number of animals which have been studied so far.

saliva is not drastically reduced on entering the rumen and is ample for its function as a buffer.

The carbon dioxide content and pH *of parotid saliva.* The number of sheep whose parotid saliva could be studied in such detail as reported in Table 4 was limited, but it was possible to obtain single samples sufficient for the determination of the carbon dioxide content and of the pH from seven other sheep which were being used for acute experiments under spinal anaesthesia, and thus to obtain more representative figures for these values. The results are given in the second and third columns of Table 6. The carbon dioxide content of these samples was fairly uniform (average 237 ml./100 ml.) and of the same order as reported for sheep 4, Table 4. The average pH was 8·23.

Rate of secretion and daily output of parotid saliva

During the experiments on sheep with parotid cannulae, frequent observations were made on the rate of secretion of the saliva. Some of the data are included in Table 4 and some additional results are given in Table 7. Both eating and rumination

Table 6. *The* CO_2 *content and the* pH *of pure parotid saliva of sheep*

Sample from ewe anaesthetized on	CO_2 (ml./100 ml.)	pH		
		Found, at room temperature	Calculated, for	
			15°	38°
24. i. 44	277	8·30	8·26	8·16
26. i. 44	241	8·46	8·20	8·10
29. i. 44	253	8·23	8·22	8·12
2. ii. 44	227	8·05	8·17	8·07
29. ii. 44	176	8·11	8·06	7·96
1. iii. 44	254	8·14	8·22	8·12
3. iii. 44	232	8·35	8·18	8·08
Average	237	8·23	8·19	8·09

On flowing into the rumen, the mixed saliva becomes diluted with the food and water of the diet and partly neutralized by the acids produced by fermentation in the rumen. The carbon dioxide content of the rumen liquor may therefore be expected to show considerable variation in relation to the diet of the animal and the time since feeding. However, a few samples of rumen liquor were analyzed for comparison with the mixed saliva. The results showed values of 40–170 ml./100 ml. from which it appears that the bicarbonate content of the mixed

brought about a considerable increase in the rate of secretion. The mere sight of food, on the other hand, caused no psychological stimulation. The effect of pilocarpine was not apparent until 30 mg. had been given intravenously. When 5 grains Nembutal was given intravenously, this was sufficient to cause the sheep to stagger on its feet, but appeared to have only a transient effect on the rate of secretion.

It was possible in these experiments to collect the saliva from the cannulated parotid of some of the sheep for considerable periods and thus to obtain a reliable estimate of the daily output. The results are summarized in Table 8. The volume from one gland was 930–1840 (average 1310) ml./24 hr. At slaughter of sheep 6, Table 8, the cannulated parotid gland was found to weigh 35 g. As it secreted 930 ml. in 24 hr., its activity can be expressed as 27 ml./24 hr./g. of tissue.

* Sheep 2, Table 4 and sheep 1 and 2, Table 5, had well established rumen fistulas. As the carbon dioxide content of the saliva from these animals was particularly low, one wonders whether the rumen fistulas had made them abnormal in this respect. Sheep 6, Table 4, however, possessed a rumen fistula for years but it had become closed at the time of the salivary studies; the saliva of this sheep had a normal carbon dioxide content.

Table 7. *Observations on the rate of parotid secretion in the sheep*

Sheep		Time to secrete 10 ml. samples (a)	(b)
No.	Condition	min. sec.	min. sec.
4	Idle	8 0	7 0
	Ruminating	2 30	2 30
	Idle	8 30	6 0
	Ruminating	2 15	2 30
	Idle	10 30	—
	Ruminating	4 0	4 30
5	Idle	11 12	—
	Ruminating	4 38	4 44
4	Before injection	43 0	—
	After 2 mg. pilocarpine subcutaneously	41 0	—
	After 30 mg. pilocarpine subcutaneously	32 0	—
	After 30 mg. pilocarpine intravenously	7 0	10 30
	Before injection (5, 2, 3, 3 drops/min.)	—	—
	After 5 grains Nembutal intravenously (14, 7, 3, 5, 4, 4, 7, 6 drops/min.)	—	—

Table 8. *Volume of saliva collected from a parotid gland of sheep*

Sheep no.	ml./24 hr.
1	1100
3	1440
4	1840
5	1240
6	930
Average	1310

DISCUSSION

Mixed saliva

Comparison with human saliva. The special importance of saliva to the ruminant was emphasized in the introduction. It is therefore relevant to compare the present results for sheep with those for an animal with a simple stomach. To this end, figures quoted for the composition of mixed human saliva in a number of text-books are presented in Table 9, together with the range of the data given in Table 3. The saliva of sheep has less dry matter and calcium, a higher ash, sodium, carbon dioxide content and pH and about the same level of magnesium and chloride, as compared with human saliva. The potassium and chloride are variable in both.

Relation to function. These differences in composition are clearly related to the special role of sheep's saliva. The large amount of carbon dioxide as bicarbonate together with the high phosphate content are functionally most important for rendering the fluid an ideal buffer for the bacterial digestion that goes on in the rumen. The bicarbonate secreted each day by the parotids can be calculated from the present data to be on the average 2·73 equiv. This may be compared with a total of 63·6 g. (1·05 equiv.) of volatile acids, as acetic acid, found in the rumen of sheep by Elsden *et al.* (1946). The salivary phosphate may have a function in addition to that of a buffer, as it was suggested by Watson (1933) that it might be important in maintaining the bacterial population of the rumen. This may well be the case as van der Wath (1942) showed that the number of these organisms was stimulated by a supplement of phosphate to a diet low in this constituent.

Parotid saliva

Typical composition. The results obtained in the experiments on sheep with cannulated parotid ducts (see Table 4) have been averaged to give a typical composition for the sheep's parotid saliva shown in Table 10. In arriving at this, the data obtained on

Table 9. *Comparison of the composition of mixed saliva of sheep and of man*

Animal	Literature reference	Content (g./100 ml.) Dry matter	Ash	Content (mg./100 ml.) Na	K	Ca	Mg	P	Cl	CO_2 (ml./100 ml.)	pH
Sheep	—	1·0–1·4	0·7–0·9	370–462	16–46	1·6–3·0	0·6–1·0	37–72	25–43	117–283	8·4–8·7
Man	(Thorpe, 1938)	0·3–1·4	0·2	26	38	8	1	10	50	—	—
Man	(Shohl, 1939)	—	0·219	20	100	6	2	18	40	22–33	6·6
Man	(Fearon, 1946)	0·6	—	20–30	30–100	8	1	3–19	40–50	—	6·35–6·85

the 3rd day of the experiments and all those for sheep 2 were excluded for reasons already stated (p. 101). The composition is expressed as mg./100 ml. and m-equiv./l. In calculating the latter, it has been assumed that at the pH of the saliva, the carbon dioxide exists as HCO_3^- and the phosphorus as $HPO_4^=$.

total carbon dioxide content and the partial pressure of the carbon dioxide, provided the composition of the solution is known. The hydrogen-ion concentrations of the samples of saliva in Table 6 were therefore calculated from the carbon dioxide content (1) at room temperature for comparison with the values actually found and (2) at body temperature

Table 10. *Average composition of the parotid saliva of sheep*

Content (g./100 ml.)		Content (mg./100 ml.)							CO_2 (ml./ 100 ml.)
Dry matter	Ash	N	Na	K	Ca	Mg	P	Cl	
1·28	0·97	20	408	32	0·8	0·8	81	61	233
		Content (m-equiv./l.*)							
		177	8	0·4	0·6	52	17		104

* P assumed to exist as $HPO_4^=$; CO_2 assumed to exist as HCO_3^-. Total cations, 186; total anions, 173 m-equiv.

Completeness of the analyses. The amounts of the various ions given in Table 10 can be calculated to be equivalent to a dry matter content of 1·29 g./100 ml. This figure is in good agreement with the average dry matter actually found, viz. 1·28 g./100 ml., and indicates that no major constituent had been overlooked. On the other hand, when the results are expressed as m-equiv., it is apparent that the total cations, 186 m-equiv./l., exceed the total anions, 173 m-equiv./l. This difference might be accounted for as sulphate which exists in small amounts in the blood or as volatile acids which were shown by Barcroft, McAnally & Phillipson (1944) to occur in the blood of the ruminant, though the amount found in arterial blood was very small. Another possibility might be the presence of an organic acid, such as lactic acid, derived from the metabolism of the gland itself. The present results, however, are sufficient to enable one to suggest a formula for synthetic saliva which may prove useful to biochemists and bacteriologists wishing to simulate the conditions in the rumen of the sheep in the study of ruminant digestion. Such a formula is given in Table 11, and is based on the data in Table 10.

Table 11. *Suggested composition for synthetic saliva*

Salt	mmol./l.	g./l.
$NaHCO_3$	117	9·8
$Na_2HPO_4.12H_2O$	26	9·3
NaCl	8	0·47
KCl	8	0·57
$CaCl_2$ anhyd.	0·2	0·04
$MgCl_2$ anhyd.	0·3	0·06

Hydrogen-ion concentration. The saliva was found by potentiometric determinations to have a pH of *c.* 8·23 (Table 6), due to the large amount of bicarbonate present. The hydrogen-ion concentration may be calculated indirectly in such a solution from the to give the reaction of the saliva as secreted. The steps in the calculations, the assumptions made and the values taken for the various constants are given below.

An approximate value of the ionic strength (μ) of the saliva was calculated, assuming the typical composition given in Table 10, whence

$$\mu = \tfrac{1}{2}\Sigma m_i z_i^2 = 0{\cdot}18,$$

where m_i = molar concentration of an individual ion and z_i its valency.

The first apparent stoicheiometric dissociation constant of carbonic acid at this ionic strength was calculated from the thermodynamic ionization constant. The simplified Debye-Hückel equation for uni-univalent electrolytes in dilute solutions gives the relation of these three quantities as

$$pK' = pK - A\sqrt{\mu},$$

in which A is a constant for a given solvent. This equation was found by Hastings & Sendroy (1925) to hold for carbonic acid in solutions containing sodium bicarbonate and chloride. As the predominating ionic species in saliva are sodium and bicarbonate and as Bray (1925) found that the activity of the bicarbonate ion is different in solutions of potassium as compared with sodium salts, the above equation was employed in the present calculations, rather than any more recent but empirical equation based on measurements made with potassium salts. Values for pK_1 at the two required temperatures were taken from Shedlovsky & MacInnes (1935) and for A from MacInnes (1939). Then for carbonic acid in saliva:

$$\text{at } 18°, \quad pK_1' = 6{\cdot}43 - 0{\cdot}50\sqrt{0{\cdot}18} = 6{\cdot}22,$$
$$\text{at } 38°, \quad pK_1' = 6{\cdot}32 - 0{\cdot}52\sqrt{0{\cdot}18} = 6{\cdot}10.$$

Before the pH of the saliva can be calculated it is necessary to know the value $[H_2CO_3]$, representing the concentration of unionized carbonic acid and physically dissolved carbon dioxide, a value depending on the carbon dioxide tension. As no data could be found in the literature for the carbon dioxide tension of sheep's saliva, blood or alveolar air, the value for human arterial blood was taken, giving

$$[H_2CO_3] = 2{\cdot}4 \text{ ml./100 ml.}$$

This seemed justified for two reasons: (1) It has been shown that equilibration with human alveolar air restored the original carbon dioxide content to samples of sheep's saliva which had lost carbon dioxide through exposure to air. The differences in the partial pressure of carbon dioxide in the two species cannot therefore be very great. (2) Small differences between the assumed and actual values of $[H_2CO_3]$ will have only a small effect on the calculated pH values.

The pH of the samples of saliva were calculated from their carbon dioxide content given in Table 6, employing the Henderson-Hasselbach equation, viz.

$$pH = pK' + \log [Salt]/[Acid].$$

This becomes, when applied to the sheep's parotid saliva:

at 15°, $pH = 6{\cdot}22 + \log [\text{Total } CO_2 - 2{\cdot}4]/[2{\cdot}4]$,
at 38°, $pH = 6{\cdot}10 + \log [\text{Total } CO_2 - 2{\cdot}4]/[2{\cdot}4]$.

The calculated pH values at 15 and 38° are given in Table 6 together with the experimental data on these samples. It will be seen that the calculated values at 15° are in good agreement with those found potentiometrically at room temperature, the averages being 8·19 and 8·23, respectively. The calculated values at 38° show that the saliva is secreted at a pH of about 8·1. This value is less alkaline than many reported for ruminant saliva but is considered more accurate.

Calcium. The saliva of the sheep contains much less calcium than the blood. This is probably because in the absence of significant amounts of protein the calcium must be largely ionized and at the pH of the saliva only small amounts of calcium ions can be in equilibrium with the carbonate and phosphate present. The maximum ionized calcium possible at body temperature was calculated for a solution with the composition of sheep's parotid saliva, as follows:

The concentration of the carbonate ion was obtained from an equation derived from the negative logarithmic expressions for the first and second stages of ionization of carbonic acid:

$$pK_1' = pH + p[HCO_3^-] - p[H_2CO_3], \quad \text{(i)}$$
$$pK_2' = pH + p[CO_3^{=}] - p[HCO_3^-]. \quad \text{(ii)}$$

Adding (i) and (ii) and rearranging:

$$p[CO_3^{=}] = pK_1' + pK_2' - 2pH + p[H_2CO_3]. \quad \text{(iii)}$$

The values $pK_1' = 6{\cdot}10$ at 38° and $\mu = 0{\cdot}18$ have already been calculated. The value of pK_2' under these conditions was obtained from the equation of Hastings & Sendroy (1925):

$$pK_2' = 10{\cdot}22 - 1{\cdot}1\sqrt{\mu} = 9{\cdot}75.$$

The pH was taken as 8·1 and the apparent concentration of unionized carbonic acid again assumed to be 2·4 ml./100 ml. The latter expressed as molar quantities gives

$$[H_2CO_3] = 1{\cdot}07 \times 10^{-3},$$

and therefore $p[H_2CO_3] = 2{\cdot}97$. Substituting these values in equation (iii) gives

$$p[CO_3^{=}] = 6{\cdot}10 + 9{\cdot}75 - 2 \times 8{\cdot}1 + 2{\cdot}97 = 2{\cdot}62.$$

The equilibrium concentration of calcium ions corresponding to this amount of carbonate was calculated from the solubility product of calcium carbonate. The value of the latter at 38° was taken from Sendroy & Hastings (1927*a*):

$$pK'_{s.p.} = 8{\cdot}58 - \frac{4{\cdot}94\sqrt{\mu}}{1 + 1{\cdot}85\sqrt{\mu}} = 7{\cdot}40 \text{ at } \mu = 0{\cdot}18.$$

Then $p[Ca^{++}] = pK'_{s.p.} - p[CO_3^{=}]$
$= 7{\cdot}40 - 2{\cdot}62 = 4{\cdot}78.$

Whence $[Ca^{++}] = 1{\cdot}7 \times 10^{-5}$
$= 0{\cdot}07$ mg./100 ml.

A series of similar calculations was made for tertiary calcium phosphate, employing the values for the three dissociation constants of phosphoric acid and the solubility product of the calcium salt, given by Sendroy & Hastings (1927*a*, *b*). The equilibrium concentration of the calcium ion with the phosphate concentration found in saliva was found to be $[Ca^{++}] = 0{\cdot}1$ mg./100 ml.

It follows from the above considerations that the low salivary calcium is secondary to the high bicarbonate and phosphate contents. The values actually found are higher than the calculated figures. One can only speculate whether the discrepancy is due to experimental error in determining the small amount present, to the existence of colloidal calcium phosphate, to supersaturation or to an undissociated complex.

Comparison with sheep's serum. As the dry matter of the parotid saliva of sheep is largely mineral and the mineral constituents of such secretions must be derived from the blood, it is interesting to see how the mineral compositions of these two fluids compare. The average composition of the parotid saliva has therefore been presented in Table 12 together with figures for the composition of sheep's serum.

Table 12. *Comparison of the composition of parotid saliva and serum of sheep*

Constituent	Parotid saliva	Serum
Dry matter (g./100 ml.)	1·28	8·3
Nitrogen (mg./100 ml.)	20	865
Reducing sugars (mg./100 ml.)	Absent	105
Na (mg./100 ml.)	408	350–380*
K (mg./100 ml.)	32	30–40*
Ca (mg./100 ml.)	0·8	10–11
Mg (mg./100 ml.)	0·8	2–5
P (inorganic) (mg./100 ml.)	81	4·5–6*
Cl (mg./100 ml.)	61	370
CO_2 (ml./100 ml.)	233	56

* From Shearer & Stewart (1931); remainder from Dukes (1943).

The concentrations of the principal cations, sodium and potassium, are similar in both fluids. The concentrations of both calcium and magnesium are less in the saliva. The greatest differences, however, are shown by the anions. The carbon dioxide content of the saliva is about four times the normal alkali reserve of sheep's blood. It might be expected that the continuous loss of so much alkali from the

blood as bicarbonate would tax the ability of the sheep to maintain the acid-base balance in the blood. This loss, however, may be offset not only by reabsorption lower down in the digestive tract but also by the abomasal secretion which in the sheep is not only acid but also continuous. The phosphorus of saliva is entirely inorganic, variable in amount and has a concentration about fifteen times that of the serum inorganic phosphorus. This is in agreement with the data of Watson (1933) who found in addition that the phosphorus in the saliva appeared to depend on the inorganic phosphorus in the serum. The chloride occurs to about one-sixth of the extent of its normal level in the serum.

Nature of the secretion. It is evident from Table 12 that the parotid gland mainly secretes ionized salts. This suggests that the difference between the ionic composition of serum and saliva might be due to a Donnan effect of the serum proteins. However, it is evident by inspection of the data that the required relation for uni-univalent electrolytes, namely,

$$[\text{Base}]\,[\text{Acid}]_{\text{Saliva}} = [\text{Base}]\,[\text{Acid}]_{\text{Serum}}$$

does not hold. Hence saliva cannot be regarded as a transudate in equilibrium with the blood.

If the two fluids are in static equilibrium, the general condition must hold that there is no net gain or loss of free energy involved in the formation of the saliva. Assuming that all the constituents of the latter are derived from the serum, the associated change in free energy can be calculated without making any assumptions as to the mechanism of saliva formation.

The decrease in free energy, $-\Delta F$, for 1 l. of saliva is given by the expression

$$-\Delta F = \Sigma n RT \log_e (a_{\text{serum}}/a_{\text{saliva}}),$$

where n is the number of mol. of a constituent transferred from a very large volume of serum to 1 l. of saliva and a its activity. The ratios of the activities can be sufficiently approximated by the ratios of the molar concentrations. In calculating the latter, the median value has been employed for those serum constituents for which a range only has been quoted in Table 12. Serum calcium has been assumed to be 60% ionized. Phosphate in the saliva has been regarded as entirely $HPO_4^{=}$ and the proportion existing in this form in the serum has been calculated for pH 7·4 using the expression of Hastings & Sendroy (1927*a*) for pK_2' of phosphoric acid. The work of transference of other phosphate ions has been taken as negligible.

In this way it is estimated that

$$-\Delta F = -130 - 163 = -293 \text{ cal.};$$

the first term refers to the transference of salts and the second to the transference of water. The saliva is therefore not a dialysate in equilibrium with serum as its formation requires a small but definite supply of energy. Whether this can be obtained from the work of ultrafiltration done on the saliva or whether it must be supplied through the metabolism of the gland itself is a problem for further investigation.

SUMMARY

1. The composition of the mixed and parotid salivary secretions of the sheep has been studied.

2. The dry matter, 1·0–1·4 g./100 ml., of both fluids was mainly inorganic but contained small amounts of nitrogen, 9–36 mg./100 ml. The mixed secretion usually appeared to consist largely of parotid saliva. The concentrations of total salts in the latter, 180 m-equiv./l., and of the principal cations, sodium and potassium, were similar to those occurring in serum. The concentrations of the anions, of bicarbonate, and of phosphate and chloride, were approximately four times, fifteen times and one-sixth respectively as much as those obtained in the serum.

3. The carbon dioxide content of the parotid saliva, mixed secretion and rumen liquor was found to be 233, 25–200 and 40–170 ml./100 ml., respectively. The parotid saliva had an average pH of 8·2 and was calculated to be actually secreted with a pH of 8·1.

4. The amount of saliva formed by a single parotid gland ranged from 930 to 1840 ml. in 24 hr.

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Studies in Vitamin A

6. CONVERSION *IN VIVO* OF VITAMIN A ALDEHYDE (RETINENE$_1$) TO VITAMIN A$_1$

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Retinene$_1$ is a fairly stable protein-free substance first obtained from rhodopsin (visual purple), the photolabile chromoprotein of the dark-adapted retina. It has recently been shown to be the aldehyde of vitamin A$_1$ (Morton & Goodwin, 1944; Ball, Goodwin & Morton, 1948). Chemical methods for its preparation have been described (Hunter & Hawkins, 1944; van Dorp & Arens, 1947), and a very simple method, in which a solution of vitamin A$_1$ alcohol in light petroleum is left to stand over powdered pyrolusite in the cold (Ball *et al.* 1948), has made it easily accessible.

The present paper is concerned with the fate of retinene$_1$ when presented to body tissues other than the retina. In particular, it was desired to find out whether it possessed *per se* vitamin A$_1$ activity. The substance, in a preparation containing no detectable vitamin A$_1$ but some 75% of retinene$_1$, has been administered orally and parenterally to vitamin A$_1$-depleted rats. It has been found to be easily convertible to vitamin A$_1$, particularly in the gut wall.

EXPERIMENTAL

Retinene$_1$

Preparation and storage. Vitamin A$_1$ aldehyde was prepared by the method of Ball *et al.* (1948). The material used had been purified by chromatography and contained about 75% of the pure substance, but unchanged vitamin A$_1$ could not be detected spectrophotometrically. It was stored as a solution in light petroleum at 0° in the dark, under which conditions it was quite stable.

Solution in oil. A known quantity of refined ground-nut oil was added to a measured volume of the stock retinene solution in light petroleum. The volatile solvent was removed by warming under reduced pressure. The resulting solution in oil was used for oral administration.

Solution in propylene glycol. A similarly prepared solution in propylene glycol was administered intraperitoneally.

Determination. The amounts of retinene$_1$ administered were determined by spectroscopic methods, using the absorption maxima at 368 mμ. (*cyclo*hexane solution) and 664 mμ. ($SbCl_3$ colour test) following Ball *et al.* (1948). Similar methods using the ultraviolet maxima at 326 mμ. and the visible maximum at 617 mμ. ($SbCl_3$ colour test) were used for vitamin A$_1$. For the few extracts in which vitamin A$_1$ and retinene$_1$ occurred together, the correction procedure of Morton & Stubbs (1946) was applied to the ultraviolet absorption curves so as to determine both constituents.

Animals

Preparation for tests. Twenty-five newly weaned rats were depleted of vitamin A$_1$. Because of a shortage of rice starch, the diet low in vitamin A$_1$ which was previously used (Glover, Goodwin & Morton, 1947*a*) was modified by introducing boiled potatoes in place of rice starch. The cooked potatoes contained very small amounts of provitamin A (mainly as β-carotene). Careful analyses showed that each rat may have received the equivalent of 2–3 i.u./day of provitamin A. The

diet contained enough provitamin to ward off frank deficiency, but too little to delay depletion of the small liver reserves. After 21–28 days, the rats had become almost stationary in weight, and, when the two largest animals were killed, the lipids extracted from livers and intestines gave negative Carr-Price tests for vitamin A_1.

Administration of retinene$_1$. (*a*) *Oral.* Retinene$_1$ (1–8 mg.) in oil was given either by drops from a calibrated syringe into the back of the mouth, or through a fine rubber catheter, internal vol. 0·2 ml., direct into the stomach. For the more quantitative work exactly 1 ml. of oil solution was placed in a small flask, and *c.* 0·7 ml. was withdrawn into a 1 ml. syringe, which was then at once connected to the rubber catheter which had previously been inserted into the rat's stomach. As much as possible of the oil was expelled from the syringe, and the catheter was then carefully withdrawn while still connected to the syringe. The syringe and catheter were then washed with ether and the rinsings added to the 0·3 ml. in the flask. The retinene in the flask was then determined and the amount given to the animal calculated by difference. The method was convenient for administering *c.* 0·5 ml. of oily solution. The dose of retinene$_1$ was varied by adjusting the concentration of solute rather than the volume of solution. Groups of 3–5 animals were used at each dose level, and the individuals were killed at definite intervals of time after dosing.

(*b*) *Parenteral.* Each rat in two groups of three each was given 1–5 mg. retinene$_1$ in 0·5 ml. of oil by subcutaneous injection. Two rats were given 4·2 mg. retinene$_1$ in 0·5 ml. propylene glycol by intraperitoneal injection. All eight animals grew normally and showed no ill effects.

Preparation of tissue extracts

The liver, stomach and small intestine were removed from the anaesthetized animal. The stomach and the small intestine were separately flushed with saline, and the contents with the washings were retained for examination. The tissues (e.g. liver) were ground in a mortar with *c.* 5 times their weight of anhydrous Na_2SO_4 and a little acid-washed silver sand, and thoroughly extracted with redistilled ether (Glover *et al.* 1947*a*). Retinene$_1$ is unstable in the presence of concentrated alkali, and saponification has therefore to be avoided. In the extraction of macerated and dried intestinal tissue, addition of a little ethanol promotes filtration through the sintered glass funnel (G4), which is otherwise liable to become clogged.

The contents of the stomach and intestines were transferred to a mortar and triturated with ethanol (10–20 ml.). The mixture was then extracted with ether in a separatory funnel.

The lipid extracts were always freed from ether and taken up in a known volume of chloroform or *cyclo*hexane.

RESULTS

Oral administration

In the animals which received the retinene$_1$ by mouth, the contents of the stomach and the small intestine often contained unabsorbed and unchanged vitamin A_1 aldehyde but no trace of vitamin A_1. On the other hand, the intestinal wall and the liver contained vitamin A_1 with no trace of retinene$_1$. These results indicate that the vitamin A_1 aldehyde was reduced to vitamin A_1 alcohol immediately on absorption into the intestinal mucosa. The reduction must have taken place extremely rapidly, as no trace of the retinene was detected in the extract from the intestinal tissue.

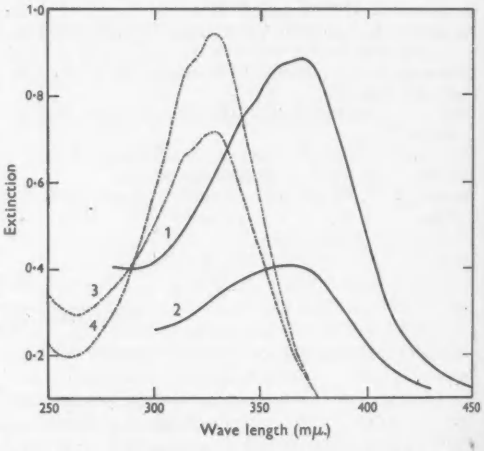

Fig. 1. Absorption spectra of lipid extracts (in *cyclo*hexane) of tissues of a rat killed 24 hr. after dosing with 8·0 mg. retinene$_1$ in 1·0 ml. oil. —— Retinene$_1$ (max. 368 mμ.): (1) stomach contents; (2) contents of small intestine. -·-· Vitamin A_1 (max. 328 mμ.): (3) gut wall; (4) liver.

Fig. 1, which records the absorption spectra of the various extracts in a typical experiment, shows the clear-cut nature of the results. The absorption curves for the liver and small intestine show maxima at 328 mμ., characteristic of vitamin A_1, whilst those for the contents of the stomach and intestine have maxima at 368 mμ., characteristic of vitamin A_1 aldehyde. Table 1 summarizes the results obtained in this series of experiments. The excellent recoveries of vitamin A_1 in the animal as shown by the quantitative experiments indicate that retinene$_1$ is stable in the lumen of the gut and its reduction to vitamin A_1 alcohol *in vivo* must be a very efficient process.

The site of absorption of the retinene$_1$ into the intestine was briefly investigated. A rat was given a dose of retinene$_1$ by stomach tube, and was killed after an interval of 15–17 hr. The small intestine was ligatured at the pyloric sphincter and caecum, and was then cut into four parts corresponding as near as could be judged with the duodenum, upper half of jejunum, lower half of jejunum and ileum. Each portion of tissue was examined for vitamin A_1 and the lumen contents checked for the presence of retinene$_1$. The results are presented in Table 2.

Table 1. *Conversion of retinene$_1$ into vitamin A$_1$ in the intestine of the rat*

Experiment	Rat no.	Dose of retinene$_1$ (μg.)	Time between dosing and test (hr.)	Retinene$_1$ recovered from: Stomach (μg.)	Retinene$_1$ recovered from: Contents of small intestine (μg.)	Vitamin A$_1$ (μg.) recovered from: Wall of small intestine	Vitamin A$_1$ (μg.) recovered from: Liver	Total recovery of retinene$_1$ + vitamin A$_1$: (μg.)	Total recovery of retinene$_1$ + vitamin A$_1$: Percentage of dose
1*	1, 2	Nil	Controls	—	—	Nil	Nil	Nil	—
	3	4000	24	Nil	Nil	551	1072	1623	41
2*	4	8000	4	4610	91	18	98	4817	60
	5	8000	7·5	4183	4	80	438	4705	59
	6	8000	16	2063	240	141	1619	4063	51
	7	8000	24	799	141	724	2405	4069	51
3*	8	2000	5	152	69	103	518	842	42
	9	2000	10	4	2	60	557	623	31
	10	2000	15	21	0	93	689	803	40
	11	2000	20	1	0	47	725	773	39
	12	2000	25	1	0	43	539	583	29
4†	13	1054	5	821	0	10	64	895	85
	14	1887	8	1080	0	126	545	1751	93
	15	1732	15	0	4	175	1070	1249	72

* Retinene administered orally as drops of oil solution.
† Retinene introduced by stomach tube.

Table 2. *Distribution of the absorption of retinene$_1$ along the small intestine of a rat killed 17 hr. after receiving 1 mg. in oil by stomach tube*

	Retinene$_1$ in intestinal contents	Vitamin A$_1$ in intestinal wall (μg.)
Duodenum	0	6
Jejunum: Upper half	0	15
Lower half	0	38
Ileum	Trace	129

It was further of interest to determine if the vitamin A$_1$ formed in the gut wall became esterified on its way into the blood stream. Light petroleum solutions of the lipid extracts from the intestinal tissue of rats 14 and 15 were examined chromatographically using a bone-meal column. In the extract from rat 14 there were 6 parts of esterified vitamin A$_1$ to 1 part of free vitamin A$_1$; and in rat 15, 3 parts of ester to 1 part of alcohol.

Parenteral administration

The three vitamin A$_1$-depleted rats in the first group injected with 4·2 mg. of retinene concentrate dissolved in 0·5 ml. of refined ground-nut oil were killed after intervals of 1 day, 5 days and 5 weeks respectively after dosing. In each case *only* vitamin A$_1$ was found in the liver. This suggested, on first thought, that the liver is the locus of conversion of injected retinene$_1$, but an examination of the site of injection proved otherwise. The surrounding subcutaneous tissues were not examined in detail for rats 17 and 18, but from their bright yellow colour obviously contained large amounts of unabsorbed retinene$_1$. The tissue near the point of injection in rat 19 had in 5 weeks become pale yellow. The pigmented portion of tissue was therefore dissected out and extracted. Analysis of the extract by the Carr-Price reaction showed that *only* vitamin A$_1$ was present. There was no trace of a reaction product with an absorption band at 664 mμ., i.e. retinene$_1$ was absent. This observation clearly indicates the presence in the subcutaneous tissue of an enzyme capable of converting vitamin A$_1$ aldehyde into vitamin A$_1$ alcohol.

The rats in the second group of three were each given 3·5 mg. of retinene$_1$ concentrate. Two of the animals were killed 3 weeks after dosing and the third 4 weeks later. The subcutaneous tissue and liver were in each case examined as before for residual retinene$_1$ and for vitamin A$_1$. The results are summarized in Table 3 and confirm the conclusion already reached. In the case of rat 21, both retinene$_1$ and vitamin A$_1$ were found in the subcutaneous tissue. The ultraviolet absorption spectrum of the extract in *cyclo*hexane is recorded in Fig. 2 (curve 1). Using the vitamin A-correction procedure (Morton & Stubbs, 1946), the true contribution of vitamin A$_1$ in the extract was calculated (curve 2). Subtracted from the whole (curve 1) this gave the absorption due to retinene$_1$ plus some irrelevant absorption (curve 3). The co-existence of retinene$_1$ and vitamin A$_1$ in the subcutaneous tissue is clear proof that the reduction of the aldehyde to the alcohol can take place there.

Table 3. *Reduction of retinene$_1$ (vitamin A_1 aldehyde) to vitamin A_1 alcohol in subcutaneous tissue of vitamin A_1-depleted rats, esterification* in situ *and liver storage*

Experiment	Rat no.	Dose of retinene$_1$ injected in oil (μg.)	Time between injection and test (days)	Recovery from subcutaneous tissue: Retinene$_1$ (μg.)	Vitamin A_1 (μg.): Total	Ester	Free	Vitamin A_1 in liver (μg.)
6	17	4200	1	Observed as deeply	—	—	—	54
	18	4200	5	stained tissue	—	—	—	600
	19	4200	35	0	446	—	—	1518
7	20	3500	21	0	311	214	71	739
	21	3500	21	291	714	631	71	1264
	22	3500	49	0	200	190	200	1175

Portions of the subcutaneous tissue extracts from this series of rats were dissolved in light petroleum, and examined chromatographically using a bone-meal column. A considerable portion of the vitamin A_1 was found in the esterified form. Thus, the subcutaneous tissue fluid contains, in addition to a reductase, an esterase or lipase capable of esterifying free vitamin A_1. This is comparable to the findings of Schönheimer & Yuasa (1929), who had previously noted that free cholesterol injected subcutaneously becomes esterified on absorption. As with the administration of retinene$_1$ by mouth there is no indication that the liver has any opportunity to effect the reduction.

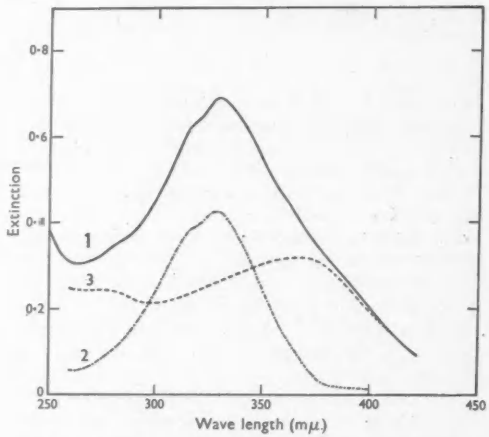

Fig. 2. Absorption spectrum (1) of the lipid extract (in *cyclo*hexane) from the subcutaneous tissue of a rat, 21 days after injection with 3·5 mg. retinene$_1$ in 0·5 ml. ground-nut oil, together with the corrected vitamin A_1 curve (2) and difference curve (3) with maximum at 368 mμ. (retinene$_1$).

With intraperitoneal administration the result was much the same. The livers of the two rats, killed 24 and 36 hr. after injection, were found to contain 491 and 635 μg. of vitamin A_1 respectively. Unabsorbed retinene$_1$ was observed in the peritoneum.

DISCUSSION

In Exp. 3, Table 1, where smaller doses of retinene$_1$ were administered (rats 8–12), the amount of vitamin A_1 in the gut wall diminished rapidly after absorption from the lumen was complete. This fact, together with the failure to detect retinene$_1$ in either the gut wall or the liver, shows that the retinene$_1$ is immediately converted to vitamin A_1 on absorption into the gut.

The accumulation of vitamin A_1 in the gut wall during active absorption (rats 4–7, Table 1) shows that entry of retinene$_1$ into the mucosa, and its conversion to vitamin A_1, are rapid processes compared with the transport of the vitamin away from the gut wall.

The mode of absorption of retinene$_1$ appears to be very similar to that of vitamin A_1 (Popper & Volk, 1944). Absorption can occur readily along the whole length of the small intestine, but normally does so in the duodenum. In the experiment referred to in Table 2 the retinene$_1$ in 0·5 ml. oil was fed to the fasting animal by stomach tube, and 17 hr. after dosing a small amount of unabsorbed fat containing a little retinene$_1$ had passed well down into the ileum. Under these conditions retinene$_1$ is apparently very stable in the digestive fluids. This was confirmed by the excellent recovery of vitamin A_1 in rats 13 and 14, Table 1, which received accurately measured doses of retinene$_1$.

In these quantitative experiments, where retinene$_1$ was fed by stomach tube, the recovery as vitamin A_1 was exceedingly high (70–90 %). This is very much better than the recovery expected after dosing with vitamin A_1 itself. According to Reifman, Hallman & Deuel (1943) the recovery of vitamin A_1 at low dosage levels is only *c.* 30 %. Gray & Cawley (1942) recovered from 40 to 70 % of different preparations of vitamin A_1 administered to rats in moderate daily doses (*c.* 100–500 i.u.). A control experiment was carried out to estimate the recovery of single doses of vitamin A_1 ester given by stomach tube at doses matching those in Exp. 4, Table 1. Each of four young rats (not vitamin A_1-depleted but with low

Table 4. *Recovery of vitamin A$_1$ ester administered in single doses by stomach tube*

Rat no.	Dose of vitamin A$_1$ in 0·5 ml. oil (μg.)	Vitamin A$_1$ recovered from: Intestine (μg.)	Vitamin A$_1$ recovered from: Liver (μg.)	Percentage of dose recovered: Total	Percentage of dose recovered: Liver only
23	1513	22	519	35·7	34·3
24	1699	14	267	16·5	15·7
25	1451	20	482	34·6	33·2
26	1686	38	579	36·6	34·3

liver stores, 30 μg./rat) was dosed with *c.* 1·5 mg. of vitamin A$_1$ ester concentrate in 0·5 ml. oil. The recovery of the vitamin from the animals killed 25 hr. after dosing ranged from 17 to 37 % (Table 4). It is worth noting, therefore, that if large liver stores are a therapeutic objective, retinene$_1$ is better than vitamin A$_1$ for attaining that end.

The absorption of retinene$_1$ from the subcutaneous tissue resembles that from the gut wall, except that it is very much slower. Retinene$_1$ has been detected 21 days after subcutaneous injection and vitamin A$_1$ after as long as 49 days. This is not unexpected, as the turn-over of tissue fluid (lymph) in a small piece of skin is much slower than in the intestinal wall, which is specially adapted for absorption. The fact that the vitamin A$_1$ alcohol, formed from the retinene$_1$ in the skin, underwent esterification before absorption is suggestive that the latter took place mainly via the lymphatic system as from the gut wall. The similarity in the modes of absorption from the skin and the intestine suggest that the enzymes, responsible for the reduction and esterification processes, are widespread in the body tissues.

Bliss (1947) has reported the isolation of a soluble protein from frogs' retinas capable of reducing retinene to vitamin A. Now that tissues other than the retina have been shown to contain the same or a similar enzyme, it may be possible to learn more regarding the nature of the enzyme in the eye. It is probably a reductase, because (*a*) the excellent recovery of vitamin A$_1$ from retinene$_1$ suggests that one molecule of the aldehyde gives rise to one molecule of the alcohol, and (*b*) it is not likely to be an aldehyde mutase which catalyzes reactions of the Cannizzaro type, producing the alcohol and the acid, since vitamin A$_1$ aldehyde contains an activated H atom in the α-position to the carbonyl group. The fact that the aldehyde is reduced easily to the alcohol, instead of being oxidized to the acid, favours the view that the alcohol grouping is necessary for vitamin A$_1$ activity.

The oxidation of β-carotene *in vitro* yields a small amount of retinene$_1$ (Hunter & Hawkins, 1944), which can be reduced to vitamin A$_1$ in a subsequent stage.

The recent work of Sexton, Mehl & Deuel (1946), Glover *et al.* (1947*b*), Thompson, Ganguly & Kon (1947), Mattson, Mehl & Deuel (1947), and Wiese, Mehl & Deuel (1947) has shown that the small intestine is the main site of the conversion of β-carotene into vitamin A$_1$. The earlier work of Goodwin, Dewar & Gregory (1946) also suggests that in the goat the intestine is capable of carrying out the conversion. In the light of all this evidence, together with the present observations, there is no longer any difficulty in accepting the suggestion that the transformation of β-carotene to vitamin A *in vivo* can take place by oxidation of the former to vitamin A$_1$ aldehyde, which is subsequently reduced to the vitamin A$_1$ alcohol. Such a process of oxidation and reduction is more probable than a hydrolytic fission.

SUMMARY

1. Retinene$_1$ (vitamin A$_1$ aldehyde) administered to rats orally, subcutaneously, or intraperitoneally is converted into vitamin A$_1$ during absorption. This suggests that the alcohol group (either free or esterified) of vitamin A$_1$ is essential for its transport and storage.

2. After oral administration the conversion takes place in the gut, and after subcutaneous injection in the subcutaneous tissues. The site of conversion after intraperitoneal injection is uncertain.

3. There is no direct evidence that the liver can convert retinene$_1$ to vitamin A$_1$.

4. The conversion represents a simple reduction of the aldehyde.

5. The extracellular spaces of subcutaneous tissue contain an enzyme capable of esterifying vitamin A$_1$ alcohol.

6. It is suggested that the transformation of β-carotene into vitamin A$_1$ *in vivo* is more likely achieved by oxidation of the former to retinene$_1$, which is then rapidly reduced to vitamin A$_1$, rather than by hydrolytic fission.

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The Composition and Reactivity of Medullated Keratins

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One of the features which distinguishes the coarser wools from the finer merino types is the presence of a medulla. Whereas the finer wools consist only of a cortex and outer scaly structure, the coarser wools contain also a medulla. The chemistry of wool and other keratins has been fairly intensively studied, but, apart from the considerable evidence which suggests a low sulphur content, relatively little is known about the composition of the medulla. Barritt & King (1931), from a comparison of the sulphur contents of medullated and non-medullated wools, calculated that the medulla substance contained little, if any, sulphur. Jordan Lloyd & Marriott (1933) found that the medullary cells of goat hair, after separation by treatment with 4N-sodium hydroxide at room temperature, contained only 0·23 % sulphur. Bekker & King (1931) showed that the medulla dissected from porcupine quill contained less sulphur than the cortex. The absence of cystine from the medulla was inferred from the appearance of cross-sections of the quill stained with sodium plumbite.

Bailey (1937) observed that scoured samples of kemp, which is a heavily medullated fibre, and to a much lesser extent wool, had a low cystine content. One such sample of kemp contained less than 2 % of cystine. He concluded that the cystine originally present had been destroyed, probably during the scouring process. The cystine of kemp would therefore appear to be much more readily attacked by mild alkalis, i.e. the scouring liquor, than that of other keratins.

Stoves (1946 *a*, *b*) has recently studied the medulla cells of a number of animal fibres, isolating them from bleached horse hair and other keratin fibres by chemical treatment. Observation of the change in appearance of the cells when treated with a 1 % tannic acid solution, 0·1 % sodium oleate or 1 % saponin suggested that the surface of the medulla cells contained a lipoprotein-cholesterol combination. Stoves further showed that when the medulla cells were refluxed with ethanolic sodium hydroxide the solution gave a positive reaction for phosphate, whilst a chloroform extract of the solution gave a positive reaction for sterols.

In the present paper the sulphur analyses of wool and kemp which had been scoured in the laboratory, and wool which had been scoured on a commercial scale, are compared with those of the same wool which had been degreased with solvents. The results of phosphorus determinations on a number of medullated and non-medullated keratins, and the examination of hydrolysates of medulla by two-dimensional partition chromatography are also given.

METHODS

Materials. All the keratins were first extracted with benzene, then with ethanol in a Soxhlet, repeatedly rinsed in water and dried in air. The medulla cells of wool fibres were isolated by the technique of Jordan Lloyd & Marriott (1933); those of feather rhachis and porcupine quill by dissection. Kemp fibres were separated from wool fibres by hand.

Analyses. Total sulphur estimations on keratins were carried out by the Benedict-Denis method (Barritt, 1934); disulphide S and thiol S estimations by Shinohara's (1935, 1936) methods; methionine by Baernstein's (1936) volatile iodide method; phosphorus estimations by Kuttner & Lichtenstein's (1930) method (see Gortner, 1945; Burmaster, 1946). Analytical results are given as a percentage of the dry weight of the keratin. Qualitative amino-acid examination of the keratins was carried out by two-dimensional partition chromatography on paper (Consden, Gordon & Martin, 1944). The keratins were hydrolyzed for 24 hr. with 5N-HCl, excess acid being finally removed by distillation *in vacuo*. The chromatograms were run in collidine for 2 days and then in phenol-ammonia for 1 day.

Scouring methods. Part of a Radnor fleece, containing a large proportion of kemp, and a sample of 64's Cape wool were scoured in the laboratory, the tips of the Cape-wool fibres being cut off as a preliminary step. The samples were treated with a solution containing 0·2% soap and 0·1% Na_2CO_3 for 3 min. at 47°. After passing through rollers, the wool was again treated with 0·1% soap solution for 3 min. at 47°, again passed through rollers and air-dried. Samples were also taken of wool at various stages during its passage through a commercial scouring set. A 70's quality Australian greasy wool was scoured in a five-bowl set. In the first bowl, the wool was given a water steep. The second bowl contained Na_2CO_3, the third soap and Na_2CO_3, the fourth soap, the final rinse being carried out in the fifth bowl. Hourly additions of soap were made to the third and fourth bowls, and the liquors were blown back at the end of a run in the usual way. The temperature of each bowl was taken during the run, and samples of the liquor removed for subsequent pH determination with the glass electrode. Samples of wool were removed before scouring, as it emerged from each bowl, and after passage through the dryer. The samples were removed shortly after the start of a run, so that the liquor in the bowls was probably in the most alkaline condition and the grease content low. Before analysis, they were extracted with light petroleum, then with ethanol, rinsed in water and dried.

RESULTS

The phosphorus content of keratins

Table 1 gives the phosphorus content of a number of medullated and non-medullated keratins. None of them contains more than a very small amount of phosphorus, which is possibly derived from small amounts of suint which have not been removed by the purification procedure employed, and shown by Freney (1934) to contain phosphates. Some support for this view is given by the fact that non-medullated wools may contain as much phosphorus as medullated wools and keratins.

Table 1. *The phosphorus content of keratins*

Keratin	Phosphorus (% of dry wt.)
Blackface wool	0·020
Welsh mountain wool	0·015
Deccani wool (Poona)	0·021
Horsehair (Argentine black mane hair)	0·039
Kemp from Radnor fleece	0·022
Cape 64's merino wool*	0·021
New Zealand 56's crossbred wool*	0·023
Blackface wool medulla	<0·02
Porcupine quill cortex	0·011
Porcupine quill medulla	0·002
Hen feather	0·018

* A non-medullated keratin.

The present results enable an upper limit to be assigned to the phosphorus of keratins present as phosphatide. The medulla constitutes only a small proportion of the total weight of wool: Barritt & King (1931) found 5·5% by microscopical observations; Jordan Lloyd & Marriott (1933) 13·5% in goat hair by isolation. If wool possesses the lipoprotein-cholesterol complex only on the surface of the medullary cells, its phosphorus content should be lower than that of the medulla. Such cells, isolated from blackface wool in amounts of 5–10 mg., after digestion with sulphuric acid, contained no phosphorus detectable by the Kuttner & Lichtenstein (1930) procedure. By this method, very small amounts (<0·02% on the weight of the medulla) should have been detected.

These results are in contrast to those of Stoves (1946 *a*, *b*), who obtained a 'positive tartaric acid-ammonium molybdate-benzidine reaction' for phosphate on an extract prepared by refluxing medulla cells with 10% ethanolic sodium hydroxide for 5 hr. It is difficult to explain the difference between these two results, unless the lipoprotein-cholesterol complex is only found in a very few types of medullated fibres. Similar amounts of medulla cells of the rhachis of hen feather also contained no detectable phosphorus, and the medulla of porcupine quill only a small amount. In contrast, the cortex of the quill had a definite phosphorus content. The reverse would be expected if the medulla contained appreciable amounts of lipoprotein. It is interesting that medullary cells isolated from the rhachis of hen feather, or from porcupine quill by dissection, do not show the swelling and other reactions on treatment with tannic acid and sodium oleate observed by Stoves on medulla cells from Kolinsky fibres.

The cystine content of scoured keratins

Table 2 compares the analyses of the laboratory-scoured wool and kemp with those of the solvent-degreased specimens. The similarity of the analyses indicates that the laboratory method of scouring

causes little destruction of the combined cystine of wool and kemp. A similar conclusion can be drawn from Table 3, which gives the analyses of commercially scoured wool; the cystine sulphur and total sulphur contents of the wool after treatment in the various bowls of the scouring set are similar to those of the original wool. The cystine of wool thus seems to be stable to a commercial scouring process.

Table 2. *The sulphur analyses of laboratory-scoured and solvent-degreased wool and kemp*

(Results as percentage of protein weight)

	Total S	Cystine S	Methionine S
64's Cape wool:			
(*a*) Scoured	3·45	3·14	0·12
(*b*) Solvent degreased	3·48	3·09	0·12
Radnor kemp:			
(*a*) Scoured	3·10	2·45	0·22
(*b*) Solvent degreased	3·24	2·45	0·22

Table 3. *The sulphur analysis of wool, before, during and after a commercial scour*

	Temp. of bowl (°)	pH of bowl	Total S (%)	Cystine S (%)
Original wool	—	—	3·48	2·97
Wool emerging from: 1st bowl	52	9·85	3·53	3·02
2nd bowl	48	10·35	3·52	2·88
3rd bowl	48	10·70	3·46	2·82
4th bowl	40	9·40	3·48	2·95
5th bowl	38	8·95	3·50	2·92
Wool emerging from dryer	—	—	3·48	2·91

These results contrast with those of Bailey (1937). He found that while wool and kemp, obtained from the raw fleece which had been degreased with solvents, gave analyses in which 98–99% of the total sulphur was accounted for as cystine and methionine, only 90% of the total sulphur of scoured wool could be accounted for in this way, and only 36% in the case of scoured kemp. The present scoured kemp sample in particular does not show the strikingly large percentage of sulphur which is not present as cystine or methionine shown by Bailey's sample. Unfortunately, Bailey's analyses were carried out on different specimens of wool and kemp, the scoured and solvent-degreased samples being derived from different sources and possibly being of different types. Unless this author's kemp had received exceptionally severe damage during processing, its unusual analysis can probably be attributed to some other cause than alkali damage during scouring.

Virgin wools and other keratins which have been degreased with solvents, and show no obvious signs of damage, often contain varying amounts of sulphur which is not present as cystine or methionine. Bailey similarly found that 6% of the sulphur of edestin and 7% of the sulphur of gliadin could not be accounted for in this way. This sulphur does not seem to be derived from lanthionine, which might be formed by the action of alkali on the combined cystine, since two-dimensional partition chromatographic analysis (Consden *et al.* 1944) of such wools often shows no detectable amount of lanthionine. Schoberl & Rambacher (1940) have suggested that the extent of damage by scouring might be determined by comparing the total sulphur with the cystine content. This suggestion should be treated with caution, since their criterion cannot be upheld in the present instance.

The amino-acid composition of medulla from keratins

In view of the evidence that the medulla of wool and porcupine quill has a sulphur content different from that of the cortex, two-dimensional chromatograms were run on acid hydrolysates of medulla to obtain some idea of their amino-acid composition. The chromatogram derived from the medulla of blackface wool differed from that of normal wool. For the medulla, the spots corresponding to glutamic and aspartic acids were strong, as were those corresponding to the basic amino-acids. The leucine spot was very strong, but those of the remaining amino-acids were weak. Similar chromatographic analysis was made on the medulla of porcupine quill. The spots corresponding to glutamic acid, leucine, valine, alanine and glycine were very strong, those of the remaining amino-acids being very weak, many not being present in sufficient amount to give a readily observed spot. The chromatogram given by the cortex of the same quill (the quill tip) differed greatly from that of the medulla and closely resembled that given by normal wool. Analysis showed the medulla of a porcupine quill to contain 0·66% of disulphide + thiol S, as compared with a value of 1·47% for the cortex, which had a total sulphur content of 1·97%. (Bekker & King (1931) found that the medulla of porcupine quills had a total sulphur content of 1·24% as compared with a value of 1·50% for the cortex.)

DISCUSSION

Previous work had indicated that the medulla of keratins has a lower sulphur content than the cortex. The present results support this conclusion and provide evidence that the medulla differs in composition from the cortex with respect to other amino-acids. The cystine of a heavily medullated kemp fibre, however, considered as a whole, does not

seem to differ from that of a normal keratin in its stability to the alkalis used in scouring. On the other hand, the treatment with sodium hydroxide used in isolating the medulla from wool probably involves some chemical degradation (Jordan Lloyd & Marriott, 1933; Stoves, 1946*a*). The very low sulphur content of the medulla of goat hair, found by Jordan Lloyd & Marriott (1933), may in part be due to the destruction by alkali of any cystine present. The medulla of porcupine quill can be isolated without such treatment.

The X-ray diagram of medullary cells differs from that given by the cortex (Rudall, cited by Stoves, 1946*a*). Keratins in general give two types of X-ray diagram, wool and hair an α-keratin diagram, corresponding to folded polypeptide chains, while feather keratin gives a β-keratin diagram, corresponding to almost fully extended polypeptide chains (Astbury & Marwick, 1932). The X-ray diffraction pattern of medulla cells from porcupine quill shows the presence of β-keratin and some unidentified material; the cortex gives a well-defined α-keratin diagram (MacArthur, 1943).

The main polypeptide chains of the medulla of porcupine quill are thus in the extended condition, in contrast to the folded chains of the cortex, and it is noticeable that the amino-acids which are predominant in the former are those with non-polar, often short, side chains, i.e. glycine, alanine, valine and leucine. This might suggest that one factor favouring the α- or β-keratin structure in nature may be the proportion of amino-acids with relatively short side chains present. Astbury has pointed out that the structure of feather keratin may be thought of as intermediate between the structure of hair keratin (folded polypeptide chains) and that of silk fibroin (fully extended polypeptide chains). The latter protein contains a large proportion of short (glycine and alanine) side chains, and hence the polypeptide chains can pack closely together and assume an extended form. In α-keratin, which has a large number of polar side chains, the polypeptide chain assumes a folded configuration. As a generalization, it is possible that β-keratins contain a higher proportion of amino-acids with short non-polar side chains than α-keratins, and that the larger proportion of longer side chains favours the α-configuration. The chromatographic analysis of porcupine quill tip (α-keratin) and tortoise shell and snake skin, both of which give a β-keratin X-ray diagram, lent some support to this idea. Tortoise shell seems to be richer in glycine, alanine, valine, leucine, serine and tyrosine than porcupine quill tip, whereas snake skin was richer in glycine, alanine, serine and threonine.

The difference between the reaction of the medulla and cortex of wool fibres towards 4N-sodium hydroxide is probably largely dependent on structural differences. The cortex of porcupine quill is similarly attacked more rapidly than the medulla. When pieces of quill are immersed in 4N-sodium hydroxide at room temperature, a considerable part of the cortex has dissolved after 24 hr., leaving the medulla largely intact. In 3 days, however, a considerable part of the medulla has also dissolved. Similar differences were observed between other keratins. Thus a 56's New Zealand wool was almost completely dissolved in a few hours, while duck feathers were largely undissolved. In general, the keratin structure with the α-configuration is attacked most rapidly. It may be that the fully extended polypeptide chains of the medulla can pack closer together than those of the folded chains of the cortex, and are less accessible and less affected by reagents such as sodium hydroxide.

SUMMARY

1. The phosphorus content of a number of medullated and non-medullated keratins, and of the isolated medulla of keratins, has been measured. None contains more than traces of phosphorus.

2. The combined cystine of a medullated keratin (kemp) or a normal wool is stable to the alkali employed in scouring. Two-dimensional partition chromatography indicates that the medulla of wool and porcupine quill differs greatly from the cortex in amino-acid composition.

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Studies on the Liver Catalase of Normal and Cancerous Rats

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Although the investigation of the systemic effects of malignant tumours is likely to constitute a fruitful approach to the cancer problem, the difficulty has always been the distinction between true cancer effects and those of a secondary nature, due to the concomitant destruction or impairment of vital organs and their functions, to necrosis, infection or suppuration, to haemorrhage and similar accessory factors. One of the least ambiguous examples of a specific systemic cancer effect is the diminution of liver catalase activity in a tumour-bearing organism.

This decrease was first described by Brahn (1916), who determined the catalase concentration in the livers of human beings who had died from various forms of cancer, and was recently extensively studied by Greenstein (1942) and his associates (Greenstein & Andervont, 1942, 1943; Greenstein, Andervont & Thompson, 1942). Their work, which was carried out on rats with transplanted subcutaneous tumours and on mice with transplanted, spontaneous or induced tumours of various kinds, established the following facts: (1) The decrease of liver catalase is progressive with the growth of the tumour. The activity may fall to one tenth or one twentieth of the normal value within 2 or 3 weeks after transplantation of a rapidly growing tumour. (2) The effect is reversible and the liver catalase activity returns to normal after excision or spontaneous regression of the tumour. Thus, in rats from which a transplanted hepatoma had been removed the normal liver catalase activity was restored almost completely after 24 hr. and fully after 48 hr. On reimplantation of the tumour the liver catalase activity dropped again and was again brought back to normal by a second excision. (3) Kidney catalase activity is depressed much less than that of liver; only in mice with spontaneous mammary tumours is it lowered as much as, or more than, liver catalase. Erythrocyte catalase is apparently not affected at all. (4) The fall of catalase activity in the liver is probably due to a decrease of catalase concentration. No evidence for the presence of a catalase inhibitor could be found in tumour extracts or in the liver or serum of tumour-bearing rats. (5) The effect is specific in a twofold sense: although a few other liver enzymes are also less active as a result of a tumour elsewhere, e.g. D-amino-acid oxidase (Westphal, 1943) or arginase (Greenstein, Jenrette, Mider & White, 1941; Weil, 1935), the reduction is less striking than that of catalase. The majority of liver enzymes are unaffected. On the other hand, as far as is known at present, the catalase effect is caused only by cancer. In particular, it is not simply due to the presence of growing tissue, since pregnancy or growing implants of embryonic tissue fail to induce it.

It is obviously of great interest to obtain a better understanding of this phenomenon. 'Excluding any form of radiation from the tumour as too far fetched, the tumour may produce the effects noted either by giving off some toxic product to the circulation, or else by abstracting from the circulation some material essential to the normal maintenance of the liver catalase' (Greenstein, 1947).

The experiments to be described were designed to test the hypotheses quoted above. The elaboration of toxic products by growing tumours has often been postulated. Such an assumption is not unreasonable in the face of the extensive degradation processes accompanying an uncontrolled aggressive growth, which not only destroys surrounding normal tissues, but in many cases outstrips its own blood supply. One might therefore expect protein breakdown products, such as proteoses and polypeptides, in the circulation, and there is indeed some evidence for this (Winzler & Burk, 1944). It has been suggested that the output of protein split products by the tumour leads to a state of generalized intoxication and is the cause of the cachexia and marasmus of cancer patients (Reding, 1938). The effect of parenteral injection of foreign protein and of protein split products on the liver catalase of normal rats was therefore investigated.

If the invasiveness and unco-ordinated growth of a tumour results in augmented protein breakdown, the high rate of new growth will, on the other hand, greatly increase the demand for nitrogenous building materials. It is well known that a growing tumour will satisfy these demands without any regard for the welfare of the host, and the decrease in the concentration of certain liver enzymes may be due to lack of supplies required for their resynthesis or even to an accelerated 'melting-down' process. The extent of the latter would depend upon the supply of nutrient material, and thus upon the diet.

In spite of the severe secondary anaemia often induced by cancer, the content of iron and copper in liver and spleen is usually above normal (Sandberg, Gross & Holly, 1942). It is improbable therefore that it is lack of these ions which is responsible for the decrease in either haemoglobin or catalase. It cannot be due to simple inanition either, since starvation of normal or tumour-bearing rats (Greenstein, Jenrette & White, 1941 *b*; Miller, 1947) leads to a fall of liver catalase activity only in proportion with the decrease

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of liver proteins generally. The fall of liver catalase activity might, however, be caused by the exaggerated protein requirements of a rapidly growing tumour. Consequently, the progressive decline of liver catalase activity in two series of cancer-bearing rats maintained on a high- or a low-protein diet was studied. For the high-protein diet a content of 45% casein was chosen, as this is sufficient to ensure maximal growth, whereas the low-protein diet contained 8% casein, an amount adequate for the maintenance of body weight or even a moderate rate of growth in normal rats.

EXPERIMENTAL

Materials and methods

Animals. Rats of both sexes from two inbred strains, a black-hooded and a brown and white strain, were used at the age of 2–3 months, when they weighed c. 100–150 g. Their routine diet consisted of 'rat cubes' (North-Eastern Agricultural Co-operative Society, Aberdeen) with an approximate protein content of 20%, and of occasional supplements of green cabbage leaves. The low-protein and high-protein diets had the following composition:

	Low protein	High protein
Casein (%)	8	45
Maize starch (%)	72	35
Arachis oil (%)	15	15
Salts (Wesson, 1932) (%)	5	5

Water was added to the mixture to make a thick dough. The following vitamin supplements were dissolved in 100 ml. water: aneurin 6 mg., pyridoxin 6 mg., riboflavin 10 mg., calcium pantothenate 20 mg., inositol 200 mg., nicotinamide 200 mg., choline chloride 600 mg. The solution (5 ml./rat) was added daily to the water used for mixing with the food. Once weekly a few drops of cod-liver oil were added to the ration. The average amount consumed daily/rat was about 15 g. Drinking water was supplied *ad libitum*. The rats were put on the diet 1 week before their inoculation with the Jensen rat sarcoma.

Serum injections. Sheep serum was inactivated by heating to 56° for 0·5 hr. It was then passed through a bacteriological filter, sealed aseptically in ampoules and stored at 0°. Intraperitoneal injections of 2 ml. were given daily for 3–4 weeks, and were well tolerated. In animals killed only a few hours after injection the injected fluid was usually completely resorbed, and there was no sign of local reaction. Five rats surviving after 4 weeks therefore received an increased daily volume of 5 ml. for a further 15–30 days. The total amount injected thus varied from 45 to 210 ml. of serum per rat. Since the results showed no variation according to the total quantity or period of injections they were grouped together.

Peptone injections. A 10% solution of a commercial sample of bacteriological peptone was adjusted to pH 7·25 (glass electrode), and subsequently sterilized and stored in the same way as serum. It gave a voluminous precipitate on addition of saturated $(NH_4)_2SO_4$ solution, and presumably contained proteoses. Two ml. were administered daily by intraperitoneal injection for 40–83 days, corresponding to a total of 80–166 ml. of peptone solution. Here, again, the results were grouped together, since no difference was apparent between animals killed at the beginning and those killed at the end of the period. The peptone injections were not so well tolerated as the serum injections, resulting in loss of weight and listlessness. Reddening and hyperaemia of the peritoneal membranes were frequently observed at autopsy.

Transplantation of tumours. The Jensen rat sarcoma (J.R.S.) was used for transplantation. The original tumour was obtained from the Imperial Cancer Research Fund Laboratories. Regressions were frequent in the beginning, but diminished after a few passages in our strains. Although a few experiments were performed on rats bearing slow-growing or regressing tumours, these were not included in the results.

Preparation and standardization of liver extract. After decapitation and exsanguination of the animal, the liver was removed and a weighed amount (c. 7 g. if available) was thoroughly homogenized with a little distilled water in a glass homogenizer (Potter & Elvehjem, 1936). After adding more water, in all three times the weight of liver used, the homogenate was left in the refrigerator overnight and was then centrifuged at 2500 r.p.m. for 5 min. The supernatant liquid was diluted to a volume proportionate to the weight of tissue taken, i.e. 100 ml. for 7 g. of liver.

It is advisable to adjust the extracts to a definite protein content. For this purpose Greenstein, Jenrette & White (1941*a*) estimated the total N of the extract. We found it more convenient to use the rapid colorimetric estimation of protein with Folin & Ciocalteu's reagent. All extracts were accordingly diluted to a certain value of 'protein tyrosine', obtained by subtracting the 'non-protein tyrosine' from the 'total tyrosine'. The total tyrosine was estimated in 0·2 ml. of extract; after addition of 5N-NaOH (2 ml.) and reagent (3 ml.), the solution was made up to 50 ml. For the estimation of non-protein tyrosine, 5 ml. of a tungstic acid filtrate of the extract were used. The light absorption was determined photoelectrically with a tricolour-red filter and calibrated with a tyrosine standard. As shown in Table 1 there is a constant ratio between the protein tyrosine and the protein N.

Table 1. *Content of tyrosine and nitrogen in liver extracts of normal rats*

Extract	Total tyrosine (mg./ml.)	Total N (mg./ml.)	N/tyrosine
1	1·40	2·910	2·08
2	1·15	2·520	2·19
3	1·60	3·740	2·34
4	1·60	3·612	2·26
5	1·13	2·316	2·08
			Mean 2·19
Extract	Protein tyrosine	Protein N	N/tyrosine
1	1·280	2·826	2·21
2	1·060	2·348	2·21
3	1·442	3·491	2·42
4	1·479	3·398	2·30
5	1·040	2·167	2·13
			Mean 2·25

Estimation of catalase activity. The catalase activity was determined by measuring the rate of H_2O_2 decomposition colorimetrically with titanium sulphate (cf. Eisenberg,

1943). This reagent was prepared as follows: Anhydrous titanium dioxide (2 g.) was heated on a sand bath with 200 ml. of conc. H_2SO_4 at 155–165° until a clear solution was formed (usually 3–4 hr.). After cooling, the solution was poured into distilled water, diluted to 1 l. and filtered through Whatman no. 5 filter paper. One vol. of the reagent was mixed with 9 vol. of H_2O_2 solution containing 0·005–0·05 mg./ml., and the ensuing colour, which was stable for at least 24 hr., was measured photoelectrically with a blue filter. A calibration curve was determined with a solution of H_2O_2, standardized by $KMnO_4$ titration.

If the concentration of phosphate buffer in the yellow solution of pertitanic acid is higher than 0·01 M, a turbidity may appear. This can easily be avoided by the addition of 0·1 vol. of 10 N-H_2SO_4 without in any way affecting the colour development. In practice this was seldom necessary, since the H_2O_2 concentration was usually sufficiently high to require further dilution.

The catalase concentration of a liver extract was tested at three different concentration levels at 0°. The H_2O_2 concentration was 0·02 N in the majority of experiments; the solution also contained 0·04 M-phosphate buffer, pH 6·8. At time $t=0$, 1 ml. of enzyme solution of required strength was added to 50 or 100 ml. of H_2O_2-phosphate buffer solution in an ice bath. After 3, 6, 9 and 12 min., sometimes also after 1 min., samples of 5 or 10 ml. were withdrawn and rapidly mixed with 0·1 vol. of 30% (w/v) trichloroacetic acid. A sample of the filtered solution was used for the colorimetric assay. The initial value of H_2O_2 concentration was determined after adding 1 ml. of enzyme solution to a sample containing 50 ml. of H_2O_2-buffer solution + 5 ml. of 30% trichloroacetic acid and filtering. The monomolecular reaction constant was calculated by the formula

$$k=\frac{2\cdot3}{t}\log\frac{[H_2O_2]_{\text{initial}}}{[H_2O_2]_{t\text{ min.}}}.$$

Haemoglobin. This was estimated by the Haldane method.

RESULTS

Effects of high- and low-protein diets on growth. The growth rate of tumour-bearing rats on the high- or low-protein diet was determined by weighing them before transplantation, and again at death, after excision of the tumour which was weighed separately. From the data in Table 2 the following facts emerge: (1) The growth of the tumour is accompanied by a fall of the body weight of the host even on a high-protein diet, but this is significantly more severe in the rats on the low-protein diet. (2) The rate of tumour growth is significantly greater on the high- than on the low-protein diet. (3) The faster rate of tumour growth more than compensates for the loss of body weight in the high-protein series, whereas the animals on the low-protein diet show not only a fall of body weight, but also of total weight. (4) If tumour weight is plotted against days, an exponential curve is obtained, rising steeply as the tumours become older. If log (tumour weight) is plotted against days the points are grouped about a straight line (Fig. 1), the slope of which can be calculated by the method of least squares. The slope of the high-protein curve is significantly steeper than that of the low-protein curve, indicating a more rapid rate

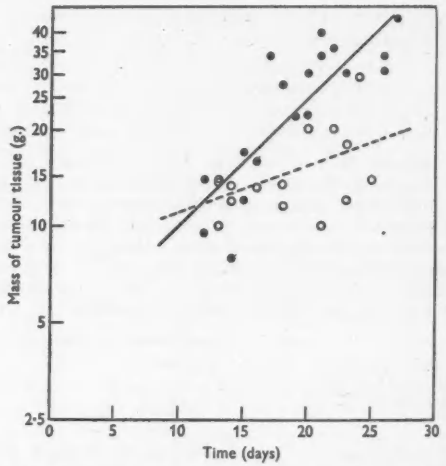

Fig. 1. Regression (statistical) of tumour weight with time. High-protein diet: dots and full line. Low-protein diet: circles and broken line. The straight lines are the linear regression lines.

Table 2. *Effect of high- and low-protein diets on body weight and tumour growth*

(Figures represent mean values and their standard errors.)

	Low-protein diet	High-protein diet	P*
No. of observations	15	18	—
Days after transplantation	18·5	19·1	—
Final body weight (% of initial value)	77·0 ±2·64	90·5 ±2·60	<0·01
Tumour growth (g./days)	0·82±0·051	1·30±0·091	<0·01
Total weight at death (body + tumour; % of initial value)	91·5 ±2·28	109·1 ±1·71	<0·01
Regression coefficients			
Log (tumour weight) on days	0·0139±0·00688	0·0393±0·00675	0·015
Log (body weight)/(tumour weight) on days	−0·0315±0·00629	−0·0494±0·00473	0·03

* P = probability of difference being due to chance.

Table 3. *Protein tyrosine content of liver extracts*

Exp.	Days after transplantation (mean)	No. of observations	Protein tyrosine (mean) (mg./ml.)	Standard error of mean
	Normal rats			
Cube diet	—	17	0·922	0·0178
High-protein diet	—	6	0·975	0·0375
Low-protein diet	—	8	0·833	0·0202
Injected with sheep serum	—	10	0·896	0·0200
Injected with peptone	—	5	0·923	0·0285
	Tumour-bearing rats			
Cube diet	22·70	19	0·710	0·0227
High-protein diet	19·26	20	0·697	0·0160
Low-protein diet	18·14	14	0·655	0·0182

of growth. (5) When the logarithm of the ratio (body weight)/(tumour weight) is plotted against days and the linear regression curve calculated

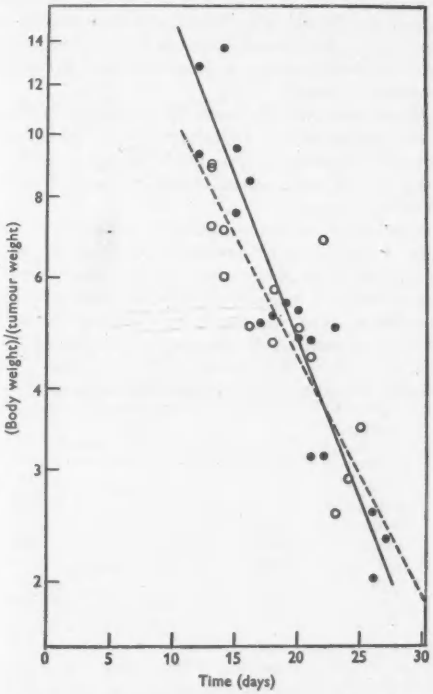

Fig. 2. Regression of ratio (body weight)/(tumour weight) on days after transplantation. High-protein diet: dots and full line. Low-protein diet: circles and broken line. The straight lines are the linear regression lines.

(Fig. 2), it is found that the difference between the high- and low-protein series, though still probably significant, is less than that between the curves representing tumour growth rates. This indicates that the faster tumour growth on the high-protein diet is to some extent balanced by a smaller loss of body weight.

Changes in protein tyrosine of liver. (*a*) *Normal rats.* The concentration of protein tyrosine in liver extracts of normal rats on the standard diet showed very little individual variation. It was not significantly raised either by a high-protein diet or by injections of serum or peptone (Table 3). On the other hand, there was a slight decrease in the rats on the low-protein diet, the difference from the standard series being statistically significant ($P < 0{\cdot}01$). The animals on the high- or low-protein diets which were used for these analyses had previously been inoculated with J.R.S., but the tumours had failed to grow or had regressed. The analyses were performed 4–5 weeks after the start of the diets and not less than 1–2 weeks after the complete disappearance of tumours.

(*b*) *Tumour rats.* The presence of a growing tumour invariably caused a considerable decrease of the protein-tyrosine concentration of liver extracts, which was not significantly affected by the protein level of the diet.

Table 4. *Haemoglobin concentration*

	No. of observations	Mean Hb (%)
Normal rats		
Cube diet	8	79·6
High-protein diet	6	85
Low-protein diet	8	76·6
Tumour rats		
Cube diet	11	49·7
High-protein diet	16	66·1
Low-protein diet	14	54·6

Haemoglobin. Estimations of haemoglobin were carried out in a number of cases and the results are shown in Table 4. There is a marked drop in tumour-bearing rats, as is well known (cf. Taylor & Pollack, 1942). Whether the differences between the high- and low-protein series are significant seems doubtful in view of the high margin of error of the method.

There were, however, indications of a correlation between the degree of secondary anaemia and the size and age of the tumour.

The catalase activity of liver extracts of normal and tumour-bearing rats. Catalase was measured at three concentration levels after 3, 6, 9 and 12 min. During this time the activity gradually diminished by 10–20%. In most experiments the enzyme concentrations were varied in the proportion of 1 : 2 : 4, the actual values depending on the presence of a tumour and its size. A value for the monomolecular reaction constant of *c.* 0·1 with the highest and of *c.* 0·025 with the lowest enzyme concentration was sought. With extracts of normal rats, final concentrations of 0·4–0·1 μg./ml. protein tyrosine were required; 1 ml. of extract containing 20–25 μg./ml. protein tyrosine was added to 50 ml. of substrate solution. With tumour rats, these concentrations had to be increased 5–10-fold. The H_2O_2 concentration in these experiments was 0·02 N throughout.

There was a very satisfactory proportionality of k (the reaction constant) with enzyme concentration (Table 5), unaffected by the relative substrate concentration. In a number of experiments the enzyme concentration was varied in the proportion of 1 : 10 : 100, to test for a possible inhibitory effect of serum or peptone injections which might have manifested itself only at high enzyme concentrations. But the fairly close proportionality of k with enzyme concentration, even over this extreme range, makes the existence of such an effect very unlikely. The ratio of substrate/enzyme concentration was kept constant here in order to avoid an unequal degree of enzyme inhibition by excess substrate.

The satisfactory proportionality between enzyme activity and concentration, at any rate over a limited range, makes it possible to express enzyme units in terms of activity and protein content. Catalase concentration has therefore been expressed in arbitrary units defined by the ratio k/mg. protein tyrosine/ml. The mean value of the results obtained with the three different enzyme concentrations was taken as the unit content of a given extract.

Catalase concentration in the liver of non-tumour rats. The concentration of catalase in the liver of normal rats is fairly constant if extracts of equal protein concentration are compared. It is not significantly changed either by high- or low-protein diets or by injections of peptone solutions (Table 6). However, injection of sheep serum leads to a significant increase of catalase concentration. Not only the difference between serum and standard series, but also that between serum and peptone series, is significant ($P<0·01$ and $P=0·01$ respectively). This is the more remarkable as the protein concentration of liver extracts was not raised by serum injections. On the other hand, although rats on the low-protein diet show a decrease of liver protein, the ratio between protein and catalase concentrations remains constant.

Since there was no essential difference between them, the first four experiments of Table 6 were grouped together to provide a standard of comparison with the catalase concentration in the liver of tumour rats.

Catalase concentration in the liver of tumour-bearing rats. Where our experiments covered the same ground as those of Greenstein and his associates the results were in full agreement with theirs. A fall in catalase activity was found, which was progressive with the growth of the tumour (in extreme cases, to

Table 5. *Proportionality of the monomolecular reaction constant k with enzyme concentration*

Series	No. of observations	Protein tyrosine (mg./ml. liver extract)	H_2O_2 concentration (N)	Mean value of k after time (min.) 3	5	12
Normal rats, cube diet	17	0·02	0·02	0·1043	—	0·0906
		0·01	0·02	0·0520	—	0·0460
		0·005	0·02	0·0276	—	0·0232
Normal rats, injected with serum or peptone	8	0·25	0·2	1·20	1·10	—
		0·025	0·02	0·178	—	0·147
		0·0025	0·002	0·0202	—	0·0158

Table 6. *Concentration of liver catalase in normal rats*

(Values in arbitrary units/ml.; see text.)

Exp.	No. of observations	Catalase concentrations (mean values) ± standard errors of mean after time (min.) 3	12
Cube diet	17	5·21 ± 0·316	4·57 ± 0·267
High-protein diet	5	5·55 ± 0·438	4·34 ± 0·404
Low-protein diet	8	5·20 ± 0·190	4·28 ± 0·163
Injected with peptone	5	6·01 ± 0·353	4·93 ± 0·280
Injected with serum	10	7·58 ± 0·427	6·24 ± 0·282
First 4 experiments combined	35	5·37 ± 0·301	4·52 ± 0·265

only 5% of normal). There were, however, large individual variations, even when similar stages of tumour growth were compared.

If a correlation is to be sought between the size of the tumour and the concentration of liver catalase, the absolute size of the tumour might be regarded as of less importance than the tumour size relative to the size of the host, as expressed by the ratio (body weight)/(tumour weight). If catalase concentrations are plotted against this ratio, an exponential curve is again obtained, and the points are grouped along a straight line when plotted on a semi-logarithmic scale (Fig. 3). In spite of the wide scatter, the regressions of catalase concentration on the ratio of (body weight)/(tumour weight) are significantly different from zero in the high- and low-protein experiments. In the experiment with rats on the standard diet the (statistical) regression is hardly significant ($P = 0{\cdot}09$). This is probably because the points were not sufficiently spread out along the x-axis.

It is quite obvious from an inspection of Fig. 3, and is confirmed by statistical analysis of the regression coefficients (Table 7), that the effect of tumour growth on the concentration of liver catalase is not significantly influenced by the level of protein in the diet. This is so not only in the case of large tumours, when it may be doubtful whether even a high-protein diet will sufficiently meet the requirements, but also in the initial stages where an effect might be expected if lack of protein were a decisive factor.

If the concentration of liver catalase is compared in rats with a similar ratio of (body weight)/(tumour weight), but with a different growth rate, it appears that it is on the whole lower when the tumours are growing slowly or are regressing than in the case of early, fast-growing tumours (Table 8). This suggests that not only the relative size, but also the age of the tumours is of importance.

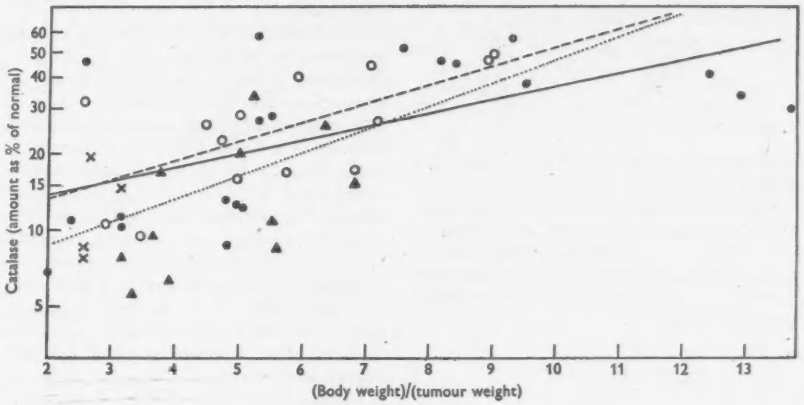

Fig. 3. Regression of liver catalase concentration (in % of normal concentration) on ratio (body weight)/(tumour weight). Standard diet: triangles and dotted line. Standard diet and serum injections: crosses. High-protein diet: dots and full line. Low-protein diet: circles and broken line. The straight lines are the linear regression lines.

Table 7. *Concentration of liver catalase in tumour-bearing rats*

Series	No. of observations	(Body weight)/(tumour weight) (mean)	Days after transplantation (mean)	Catalase units (% normal) (mean)	Regression coefficient of catalase concentration on (body weight)/(tumour weight)
Cube diet	15	4·20	22·14	13·95	$0{\cdot}0747 \pm 0{\cdot}0408$
High-protein diet	20	6·53	19·05	29·22	$0{\cdot}0517 \pm 0{\cdot}0165$
Low-protein diet	14	5·61	18·14	27·65	$0{\cdot}0734 \pm 0{\cdot}0254$

In view of the definite increase of liver catalase observed in normal rats after intraperitoneal injections of sheep serum, the effect of serum injections on tumour-bearing rats was of interest. Four rats (marked × in Fig. 3) were given daily intraperitoneal injections of 5 ml. of sheep serum, starting 11 days after transplantation when the tumours were easily palpable. The injections were continued for 8–10 days, so that a total amount of 40–50 ml. was injected. There was no obvious effect on either the rate of tumour growth or the concentration of liver

catalase. Two more series each consisting of five rats received serum injections from the fourth and fifth day after transplantation onwards. In all ten animals regression of the tumours occurred. In two control series the tumours grew in 3 out of 5 and in 3 out of 3 animals. It would be premature to draw any conclusions from the small number of animals in these experiments, especially in view of a tendency towards regression in the controls, but the results suggest an inhibitory effect of early serum injections on the growth of transplanted J.R.S. This point is being further investigated.

Table 8. *Concentration of liver catalase in tumour-bearing rats*

Days after transplantation	(Body weight)/(tumour weight)	Catalase concentration (% normal)
	Fast growing, early tumours	
13	8·9	46·9
13	9·0	49·7
12	9·3	57·3
12	12·9	33·8
14	7·06	44·4
	Slow growing or regressing tumours	
21	29·0	24·3
23	11·6	27·6
23	12·2	15·9
26	7·15	10·3
27	14·3	35·8

DISCUSSION

The fact that the presence of a growing tumour leads to a progressive fall of the concentration of liver catalase far in excess of the lowering of liver proteins generally need not necessarily be inconsistent with an unspecific inhibition of protein synthesis such as would result from the appropriation of available amino-acids by the tumour. If the rate of disintegration and regeneration were much faster for catalase than for other liver enzymes, the specific effect on catalase could be interpreted as the outcome of an unspecific inhibition of protein synthesis. There are, indeed, indications that liver catalase has a short life: (1) it has a high rate of wastage during enzymic activity; (2) a high rate of resynthesis after removal of a transplanted tumour; (3) in contrast to erythrocyte catalase, it contains a verdohaemochromogen group which, as Lemberg & Legge (1943) suggest, is due to an *in vivo* oxidation.

The experiments on the effect of high- and low-protein diets have provided no support for this mechanism. If a shortage of amino-acids were the cause, one would have expected at least a delay in the decline of catalase concentration as a result of the high-protein feeding. That the fall of body weight is considerably less severe on the high- than on the low-protein diet shows that the higher protein level is not entirely ineffectual, and the lack of response of the catalase concentration suggests that other causes than protein shortage are operating. This conclusion, however, is not yet final. An effect might appear if the rate of protein synthesis could be speeded up, e.g. by parenteral administration of essential amino-acids in high dosage. Alternatively, an excess of one or more particular amino-acids may be required. The observation that serum injections raised the level of catalase concentration in normal livers, whereas peptone injections were ineffective, may point in this direction. Peptone is particularly deficient in tryptophan, and it would be interesting therefore to study the effect of massive doses of this amino-acid.

The possibility that the lowering of catalase concentration in the liver is due to a toxic principle elaborated by the tumour and given off into the circulation also deserves further study. It is true that Greenstein (1943) found no indication for the presence of a catalase inhibitor in tumour tissue or in other tissues of cancerous animals. But the possible existence of an inhibitor of catalase synthesis or a catalyst of catalase destruction has not yet been exhaustively explored. Our experiments show that prolonged parenteral injection of foreign protein or of protein breakdown products does not duplicate the tumour effect in normal rats. A prolonged treatment of normal rats with tumour extracts might now be attempted. Greenstein (1943) carried out some experiments on these lines, but he gave only one injection of 1 or 2 ml. of extract, whereas it may be necessary to extend the injections over a period of several weeks. Moreover, his extracts, which were prepared with saline, were probably devoid of nucleoproteins, and it is feasible that the toxic factor is associated with this fraction.

SUMMARY

1. The concentration of liver catalase in normal rats and in rats bearing a transplanted Jensen rat sarcoma was determined. The rats were maintained on one of three diets: (*a*) a standard diet of rat cubes (20 % protein), (*b*) a high-protein diet (45 % casein), (*c*) a low-protein diet (8 % casein). Additional experiments were performed on rats which received daily intraperitoneal injections of sheep serum or of a 10 % solution of peptone for several weeks.

2. The high-protein diet caused a significantly faster growth of the tumours and a smaller loss of body weight in tumour-bearing rats than the low-protein diet. The protein content of the liver was slightly reduced in normal rats on the low-protein diet; it was considerably lowered in all tumour-bearing rats, irrespective of diet.

3. The catalase concentration in normal rat liver extracts of equal protein content was not significantly changed, either by different protein levels in the diet or by peptone injections. It was raised, however, by serum injections.

4. In confirmation of the work of Greenstein and his group (1941–3) it was found that the growth of a transplanted tumour leads to a fall of liver catalase concentration which, in extreme cases, may reach 5% of normal. There was a very marked individual scatter even in similar stages of tumour growth.

5. The catalase concentration when plotted against (body weight)/(tumour weight) was found to follow an exponential curve.

6. The protein level of the diet had no significant effect on the course of the process, even in the initial stages. Serum injections had no effect on tumour-bearing rats when started 11 days after transplantation. When started 4 or 5 days after transplantation they were followed by regression of the tumour.

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The β-Glucuronidase Activity of Ox Spleen and the Assay of β-Glucuronidase Preparations

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The existence in animal tissues of an enzyme which hydrolyzes β-glucuronides was first reported by Sera (1915). In 1934, Masamune described a method of preparation of active extracts of this enzyme from ox kidney having pH optima for the hydrolysis of menthylglucuronide and phenylglucuronide in citrate buffers at 5·3 and 5·3–5·6 respectively.

Oshima (1934) reported data for the glucuronidase content of various tissues of the dog and the ox, rich sources being liver, spleen, kidney, ovary, testes and thymus. In 1936 Oshima introduced improvements into the method of Masamune (1934), which were claimed to give a purer preparation. For this preparation the pH optimum for the hydrolysis of menthylglucuronide in acetate buffer was quoted as 5·0–5·2. In the work reported up to that time, little or no data were presented concerning the degree of purification or the losses at each stage of the preparation of β-glucuronidase.

The first quantitative study of the purification of ox-spleen glucuronidase was reported by Fishman (1939*a*). By employing ammonium sulphate fractionation after preliminary treatment, Fishman achieved an eightfold concentration of the enzyme and a 140-fold purification. There was, however, an overall loss of 84% by this method. Fishman (1939*b*) reported that the pH optima of his preparation in an acetate buffer were 5·0 for *l*-menthylglucuronide, 4·4 for borneolglucuronide and 4·3 for oestriol-glucuronide.

Florkin, Crismer, Duchateau & Houet (1942) also studied glucuronidase, and demonstrated the reversibility of the hydrolysis of borneolglucuronide *in vitro*.

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A much improved method of preparation of ox-spleen glucuronidase was reported by Graham (1946) in which a 400-fold concentration and a 315-fold purification of the enzyme was achieved. Graham found that a preliminary autolysis of an aqueous extract of acetone-extracted tissue caused an increase in the amount of the enzyme, and that this was accompanied by a liberation of reducing material. These facts were shown to be complicating factors in the assay of crude preparations of the enzyme. It was also demonstrated that pH control was an important factor during the separation of the enzyme from inactive protein by ammonium sulphate fractionation. Graham, however, reported no data on the pH optima or reaction kinetics of his preparation.

Talalay, Fishman & Huggins (1946) reported data concerning the hydrolysis of phenolphthalein glucuronide by fairly crude preparations of glucuronidase from pooled livers, kidneys and spleens of mice. The pH optimum in acetate buffer for this substrate was shown to be 4·5. Data on the kinetics of the reaction were also presented. Talalay *et al.* (1946) and Fishman & Talalay (1947) have used phenolphthalein glucuronide and pH 4·5 in a method of assay of the glucuronidase content of various tissues of the rat.

In the present work further data on the preparation and purification of ox-spleen glucuronidase are presented. The data reveal that two enzymes which hydrolyze *l*-menthylglucuronide are present in ox spleen, and that these two enzymes, which can be separated, have different pH optima. A modified technique for the assay of glucuronidase preparations using *l*-menthylglucuronide is presented along with assay methods employing phenylglucuronide and phenolphthalein glucuronide.

A preliminary report of this work has already appeared (Mills, 1947).

EXPERIMENTAL

Reagents

l-*Menthylglucuronide*, prepared by the biosynthetic method of Quick (1924).

Phenylglucuronide, prepared by the method of Masamune (1933) as modified by Williams (1945), m.p. 160° (uncorr.).

Phenolphthalein glucuronide, prepared by the method of Talalay *et al.* (1946).

D-*Glucurone*, prepared by the method of Williams (1940), m.p. 176–177° (uncorr.), $[\alpha]_D^{20°} +20°$.

Trichloroacetic acid (British Drug Houses Ltd.), 20% (w/v) aqueous solution.

Alkaline ferricyanide solution, 5 g. $K_3Fe(CN)_6$ (A.R.), and 10 g. anhydrous Na_2CO_3 in water to 1 l. This is stable for 1 month in a dark bottle; after that time the blank value in the reducing test increases considerably.

Sodium carbonate, 10% (w/v) aqueous solution.

Ceric sulphate, approx. 0·1 N solution, prepared according to Miller & Van Slyke (1936), and diluted 1 in 10 in N-H_2SO_4 for daily use.

Sulphuric acid, 18 N (A.R.).

Lissamine green (British Drug Houses Ltd.), 0·05% (w/v) aqueous solution.

Phenol reagent, prepared as described by Folin & Ciocalteu (1927).

All pH measurements were carried out with the glass electrode and Cambridge pH meter.

ASSAY OF GLUCURONIDASE PREPARATIONS

(*a*) *Using* l-*menthylglucuronide*

In the methods of Fishman (1939*a*) and Mills (1946) the neutralization of excess trichloroacetic acid, after the removal of precipitated protein from the enzyme digests, was accomplished by N-NaOH in the presence of phenolphthalein. This method gave rise to high blank values in the subsequent ferricyanide reduction. The method of Levvy (1946), which employs an alkaline copper tungstate deproteinization, was found to be unsatisfactory if the pH of the enzyme digest was less than 4·6. This criticism applies also to a cadmium hydroxide precipitation, a preliminary exact neutralization of the digest being necessary in both cases to give reliable results.

The most satisfactory procedure so far found involves a trichloroacetic acid deproteinization followed by addition of excess sodium carbonate. It was found that wide variations in the sodium carbonate concentration were allowable in the ferricyanide reduction (cf. Levvy, 1946).

Method. Acetate buffer (0·2 M; 1·0 ml.), 0·5 ml. of 0·02 M-*l*-menthylglucuronide solution (pH *c.* 5) and 0·5 ml. of a suitable dilution of the enzyme solution are incubated in 15 ml. centrifuge tubes for 2 hr. at 38°. The reaction is then stopped and protein removed by the addition of 1 ml. of 20% trichloroacetic acid followed by centrifugation; 2 ml. of the supernatant fluid are transferred to 4 × $\frac{5}{8}$ in. Pyrex tubes and 1 ml. of 10% Na_2CO_3 solution followed by 1 ml. of alkaline ferricyanide solution added. The tubes are closed with glass bulbs, heated in a boiling water bath for 15 min. and then cooled in running water. H_2SO_4 (18 N; 0·25 ml.) is added, followed by 1 drop of 0·05% Lissamine green solution, and the mixture titrated with *c.* 0·01 N-ceric sulphate solution using a 2 ml. micro-burette. Stirring is performed by a slow stream of N_2, and a daylight lamp is used for the titration.

The ceric sulphate is standardized against D-glucurone and results expressed as glucuronic acid by employing the appropriate correction factor. Controls are carried out using buffer and enzyme solution alone, since it was found that hydrolysis of the substrate in the absence of glucuronidase at pH 3–7 could be neglected.

By using unboiled enzyme extracts in the control tubes, interference caused by the increase in reducing material on incubation of crude extracts is avoided. Graham (1946) first demonstrated this increase in reducing material, and some typical data are presented in Fig. 1. This method does not avoid, however, the increase in enzymic activity which accompanies the increase in reducing material on incubation of such crude extracts. Neither increase occurs in extracts after precipitation with ammonium sulphate.

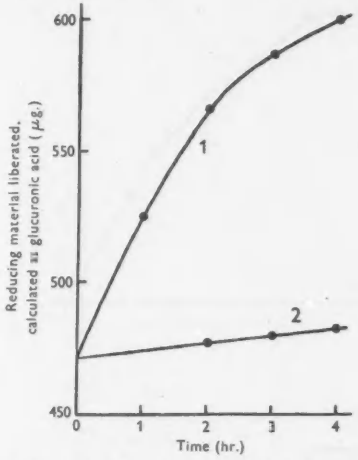

Fig. 1. Liberation of reducing material (calculated as glucuronic acid) during the incubation of 2 ml. samples of a crude spleen extract at 38°. 1, unboiled extract; 2, boiled extract.

It was also found that the presence of ammonium sulphate at a concentration of 0·05 M or less in the enzyme digests did not affect the results of assay.

The glucuronidase reaction was found to follow the equation

$$k = 1/t \log_e a/(a-x)$$

at low substrate concentrations, where t = time in hours, a = original amount of substrate and x = amount of substrate split in time t.

Unit of glucuronidase activity. The unit of glucuronidase activity (menthyl unit) was taken as the activity associated with a value of $k = 0{\cdot}1$ in the above equation, under the conditions specified.

(*b*) *Using phenylglucuronide*

The method is based upon the determination of phosphatase using phenylphosphate (King & Armstrong, 1934; Folley & Kay, 1935). Phenylglucuronide is incubated with the enzyme preparation in a buffer solution, and after a standard time the reaction stopped and the phenol estimated using the reagent of Folin & Ciocalteu (1927).

Method. Acetate buffer (0·2 M; 1·0 ml.), 0·5 ml. of 0·02 M-phenylglucuronide (Na salt) and 0·5 ml. of enzyme solution are incubated in 15 ml. centrifuge tubes for 1 hr. at 38°; 2 ml. of a 1 in 3 dilution of the Folin-Ciocalteu reagent are added and the protein precipitate removed by centrifugation. 3 ml. of the supernatant fluid are added to 3 ml. of 10% Na_2CO_3 and the mixture incubated at 38° for 40 min. Water (2 ml.) is added and the blue colour estimated in the Spekker photoelectric absorptiometer using Ilford no. 608 red filters. The phenol liberated is estimated by using a calibration curve determined with phenol solutions treated as in an enzyme assay.

Controls were set up in which the buffer and substrate were incubated together for 1 hr. at 38° and then the enzyme, immediately followed by the diluted Folin reagent, added at the end of this time.

Since the liberation of phenol was found to follow a linear course with time and to be directly proportional to the enzyme concentration, the amount of phenol liberated is, therefore, directly relatable to the amount of enzyme present.

1 phenyl unit = 1 μg. phenol liberated/hr. under the above conditions.

(*c*) *Using phenolphthalein glucuronide*

The method is essentially that of Talalay *et al.* (1946) with the difference that the enzyme digests consist of 0·5 ml. of 0·2 M-acetate buffer, 0·2 ml. of 0·005 M-phenolphthalein glucuronide (Na salt) and 0·3 ml. of enzyme solution, and that protein is removed with trichloroacetic acid before bringing the pH to 10·4. The colour is estimated in the Spekker photoelectric absorptiometer using an Ilford 605 yellow-green filter.

1 phenolphthalein unit = 1 μg. phenolphthalein liberated/hr. under the above conditions.

Determination of protein nitrogen

The protein in 1 ml. samples was precipitated with 1 ml. of 20% trichloroacetic acid and the mixture heated to 80° for 10 min. The precipitate was removed by centrifugation and washed on the centrifuge with 5 ml. of 5% (w/v) trichloroacetic acid. If ammonium sulphate was present in the original sample, the washing was repeated a further four times. The protein precipitate was dissolved in 3 ml. of 18 N-sulphuric acid and the protein N estimated, after digestion, by the micro-Kjeldahl method of Ma & Zuazaga (1942), employing the Markham (1942) distillation unit.

PREPARATION OF β-GLUCURONIDASE

The finding of Graham (1946) that acetone precipitation of the enzyme from aqueous solution followed by extraction of the precipitate with water, as employed by Fishman (1939*a*), gave rise to serious

losses was confirmed in the present work. It was also found, in accordance with Graham (1946), that by extracting the tissue pulp with two successive lots of 2 vol. of acetone and subsequent extraction of the solid residue with water, one could avoid these serious losses. Ethanol precipitation, as employed influenced by both these factors. It is quite obvious therefore that the results of ammonium sulphate fractionation of protein mixtures will be significantly influenced by variations in pH and temperature, factors which are often neglected during salt fractionation of proteins.

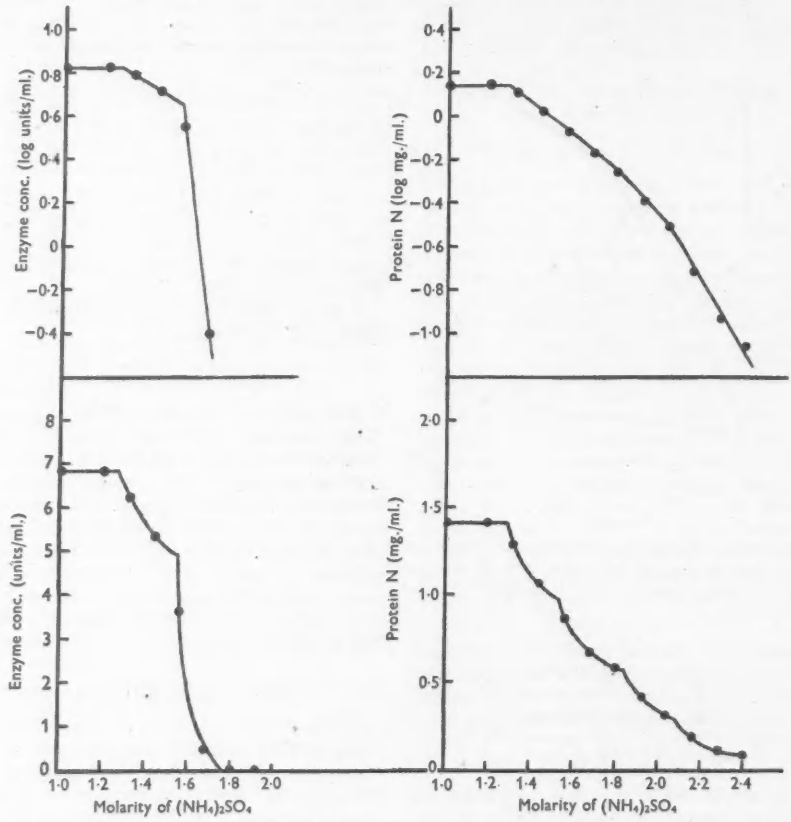

Fig. 2. Variable solvent solubility test on spleen extract at stage C of preparation, carried out at pH 5. Ammonium sulphate used as precipitant and glucuronidase assayed at pH 5 using *l*-menthylglucuronide.

by Masamune (1934) and Oshima (1936), also gave rise to as much as a 50 % loss of activity.

In the method of Fishman (1939*a*) there was no control of the pH during ammonium sulphate fractionation. Cohn (1925) has shown that the solubility of proteins in salt solutions obeys the equation

$$\log S = \beta - kI,$$

where S = solubility, I = ionic strength, and β and k are constants. Cohn found that while k is independent of temperature and pH, β is markedly

Graham (1946) took pH into account during ammonium sulphate fractionation, but did not indicate how the limits employed were derived.

In order to obtain precise data in the present work the variable solvent solubility test as defined by Falconer & Taylor (1946) was applied to crude glucuronidase solutions at two different pH values.

Samples (4 ml.) of a glucuronidase solution, adjusted to the requisite pH, were introduced into a series of 15 ml. centrifuge tubes and saturated $(NH_4)_2SO_4$ at the same pH added to give the required ionic strength. The tubes were

allowed to stand at 0° for 18 hr. and the precipitates, removed by centrifugation at 0°, were dissolved in water and assayed at pH 5 for glucuronidase activity using *l*-menthylglucuronide. Protein N determinations were carried out on the supernatant solutions.

The data obtained at pH 5·0 and 7·0 (Figs. 2 and 3 respectively) reveal that two proteins exist in spleen extracts, both having glucuronidase activity. Falconer & Taylor (1947) indicated methods of separation of two phases (A and B) based upon determination of the constants β and k. They stated that if for two fractions k_A is greater than k_B then a dilution of the original solution aids separation of phase A in a pure form. If k_B is greater than k_A then concentration aids in obtaining A in pure form. Also conditions of temperature and pH which make $\beta_A - \beta_B$ a maximum are optimum for separation.

These constants must be determined on the pure phases to give absolute values, but the direction of difference can be obtained from the solubility test. If in the present work the fraction precipitated at lower salt concentration gives k_A and the other fraction k_B then it is obvious from the data that k_B is greater than k_A.

A method of purification of glucuronidase was worked out using the data obtained above. Throughout the procedure which follows, the pH was controlled at each stage by means of a glass electrode, and all operations requiring temperature control were carried out in a cold room at 0°.

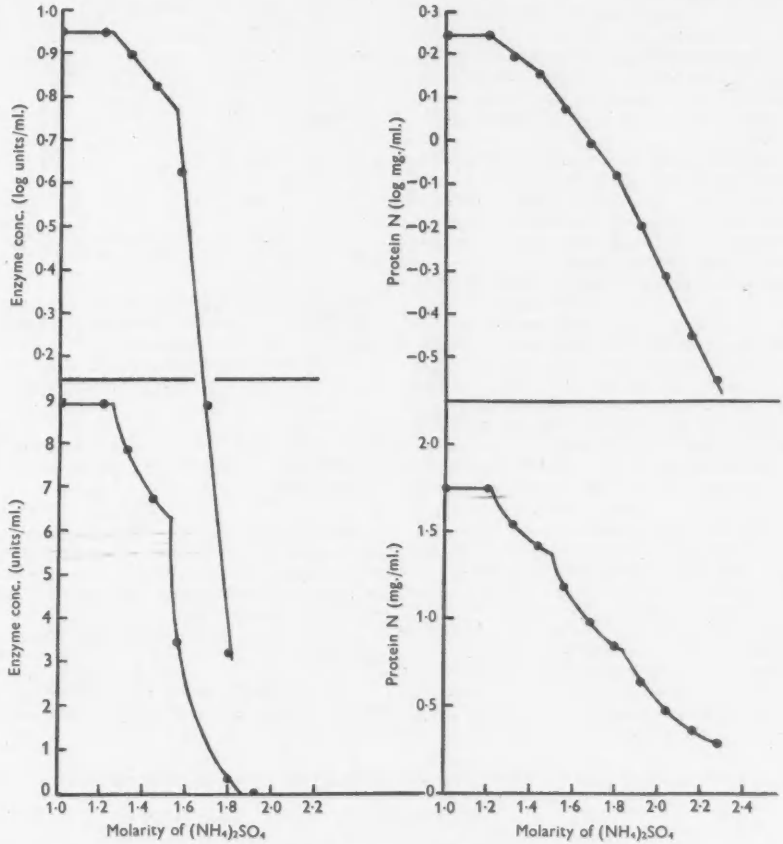

Fig. 3. Variable solvent solubility test on spleen extract at stage C of preparation, carried out at pH 7. Details as for Fig. 2.

Stage A. Fresh ox spleens were stripped of fat and finely minced. Redistilled acetone (3 l.) was stirred into 1·5 kg. of this mince and the mixture filtered and sucked dry. The pressed filter cake was stirred with a further 3 l. of acetone and the mixture filtered with suction and pressed well. The filter cake was broken up and dried in air at room temperature for about 30 min. The spleen powder was stirred with 3 l. of water for 1 hr., strained through gauze, clarified in the Sharples super-centrifuge and dialyzed against running tap water for 24 hr. (solution A).

Stage B. Solution A was adjusted to pH 5·0 with 2N-acetic acid and M-acetate buffer (pH 5·0) added (20 ml./l.). A voluminous precipitate formed. The mixture was allowed to stand at 38° for 4 hr. and the precipitate removed in the Sharples super-centrifuge. The filtrate was finally clarified by filtration using Standard Super-Cel (Johns-Manville Co. Ltd.) (solution B).

Stage C. Solid $(NH_4)_2SO_4$ was stirred into solution B to 60% saturation and the mixture allowed to stand at 0° for 18 hr. The supernatant liquid was siphoned off and the precipitate removed by filtration using Standard Super-Cel (10 g./l.). The filter cake was sucked dry and then extracted five times with 100 ml. portions of water. The extracts were combined and dialyzed against running tap water for 24 hr. (solution C).

Stage D. Solid $(NH_4)_2SO_4$ was added to solution C to 1·2M and the pH adjusted to 5·0 with 2N-acetic acid. The mixture was allowed to stand at 0° for 6 hr., the precipitate removed at the centrifuge and discarded. To the solution $(NH_4)_2SO_4$ was added to 1·8M, the pH readjusted to 5·0 and the mixture allowed to stand at 0° for 18 hr. The precipitate was removed at the centrifuge at 0°, dissolved in water and dialyzed against running tap water for 24 hr. (solution D). This stage removes a considerable amount of inactive protein.

Stage E. Solution D (about 500 ml.) was adjusted to pH 5·0 with N-acetic acid, and saturated $(NH_4)_2SO_4$, previously adjusted to pH 5·0, was added to 1·26M. The mixture was allowed to stand at 0° for 6 hr. and the precipitate removed at the centrifuge at 0° and discarded. To the supernatant liquid saturated $(NH_4)_2SO_4$ at pH 5 was added to 1·54M, the solution allowed to stand at 0° for 18 hr., the precipitate removed at the centrifuge at 0°, dissolved in 25 ml. of water and dialyzed against running water for 24 hr. (fraction I). To the supernatant liquid from the above precipitation, $(NH_4)_2SO_4$ at pH 5·0 was added to 1·78M, the mixture allowed to stand at 0° for 18 hr., the precipitate removed at the centrifuge at 0°, dissolved in 200 ml. water, and dialyzed against running water for 24 hr. (fraction II).

Stage F. Fraction I was reprecipitated with saturated $(NH_4)_2SO_4$ at pH 5 between 1·26 and 1·52M at 0°, and the precipitate was washed on a centrifuge at 0° with 1·52M-$(NH_4)_2SO_4$ at pH 5, dissolved in the minimum amount of water and dialyzed against running water for 24 hr. Fraction II was reprecipitated from the combined supernatant liquids with saturated $(NH_4)_2SO_4$ at pH 5 between 1·56 and 1·76M at 0° and the precipitate dissolved in about 200 ml. of water and dialyzed against running water for 24 hr.

Stage G. Stage F was repeated to obtain more pure fractions.

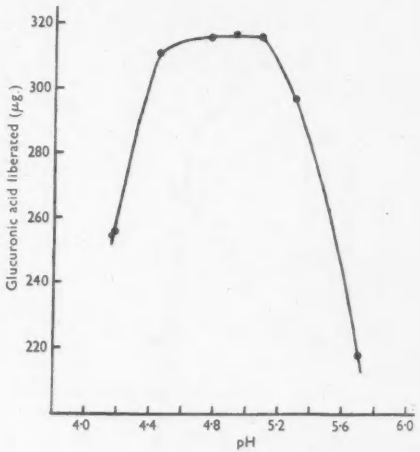

Fig. 4. pH-activity curve of spleen extract at stage D of preparation. Glucuronidase assays using *l*-menthyl-glucuronide.

Data for the activity of a typical preparation at each stage of purification are presented in Table 1.

Table 1. *Activity of β-glucuronidase preparations at various stages of purification*

Stage	Vol. (ml.)	Assays at pH 4·5		Assays at pH 5·0		Ratio units at pH 5·0 / pH 4·5
		Units*/ml.	Total units	Units*/ml.	Total units	
A	3600	3·14	11,304	2·99	10,764	0·95
B	3400	4·02	13,668	3·90	13,260	0·97
C	780	17·13	13,361	16·45	12,831	0·96
D	580	16·79	9,738	16·45	9,541	0·98
E: Fraction I	35	133·12	4,659	114·61	4,011	0·86
Fraction II	220	15·91	3,502	17·85	3,927	1·12
F: Fraction I	33	98·00	3,234	82·12	2,710	0·84
Fraction II	230	11·92	2,741	13·95	3,208	1·15
G: Fraction I	24	111·20	2,669	90·04	2,161	0·81
Fraction II	31	72·61	2,251	85·63	2,654	1·18

* Menthyl unit of glucuronidase activity, see p. 127.

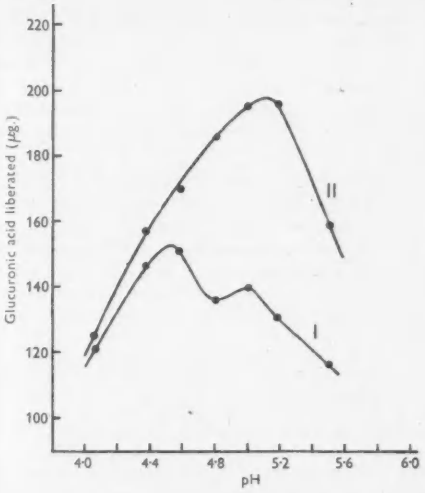

Fig. 5. pH-activity curves of fractions I and II at stage E of preparation. Glucuronidase assay using *l*-menthylglucuronide. I, fraction precipitated between 1·26 and 1·54 M-$(NH_4)_2SO_4$; II, fraction precipitated between 1·54 and 1·78 M-$(NH_4)_2SO_4$.

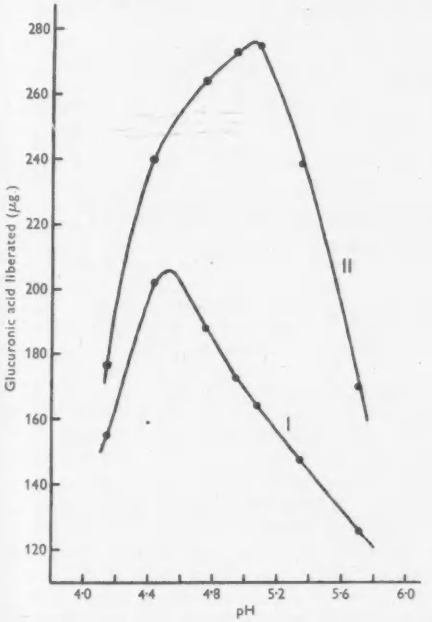

Fig. 6. pH-activity curves of fractions I and II at stage G of preparation. Glucuronidase assays using *l*-menthylglucuronide. Details as for Fig. 5.

pH-activity curves using *l*-menthylglucuronide were determined for the preparations at stages D, E and G, and typical data are presented in Figs. 4, 5 and 6 respectively. The pH-activity curve at stage D in some preparations showed evidence of two peaks; in others, the curve shown in Fig. 4 was obtained. pH-activity curves for the pure fractions were also determined using phenyl and phenolphthalein glucuronides, and the data for all three substrates are shown in Table 2.

Table 2. pH *optima of ox-spleen β-glucuronidases*

	β-Glucuronidase	
Substrate	I	II
l-Menthylglucuronide	4·5	5·0
Phenylglucuronide	4·5	5·2
Phenolphthalein glucuronide	4·5	5·2

DISCUSSION

It would appear from the work of Masamune (1934), Oshima (1936), Fishman (1939 *a*, *b*) and Talalay *et al.* (1946) that these authors considered they were dealing with a single enzyme. This conclusion may reasonably be drawn from the data they presented on the kinetics of the hydrolytic reaction. While presenting no data upon optimum pH or reaction kinetics, Graham (1946) gave no evidence which could indicate that there was more than one enzyme present in his extracts.

By the application of the variable solvent solubility test it has been possible to show the presence in spleen extracts of two proteins having β-glucuronidase activity. The fact that the two enzymes have different pH optima makes extremely difficult the interpretation of the specific property solubility test of Falconer & Taylor (1946). At pH 5 the two proteins having glucuronidase activity are precipitated by ammonium sulphate within the limits 31·5–38·5 % and 38·5–44 % saturation, these two fractions having pH optima for the hydrolysis of *l*-menthylglucuronide of 4·5 and 5·0 respectively. Fishman (1939*a*) used the limits 37–50 % saturation and Graham (1946) 36–46 % saturation with ammonium sulphate in the preparation of their enzyme extracts. It is obvious, therefore, that these workers eliminated the major portion of the enzyme having optimum pH 4·5 for the hydrolysis of *l*-menthylglucuronide. It is probable, therefore, that the data of Fishman (1939*b*) concerning pH optima and Michaelis constants may be substantially correct for one of the enzymes.

In later work Talalay *et al.* (1946) used a preparation from a mixture of livers, spleens and kidneys of mice and made no attempt at fractionation. The data presented concerning the optimum pH for the hydrolysis of phenolphthalein glucuronide (4·5) and

reaction kinetics will need revision, since no account is taken of the dual nature of the spleen enzyme and any possible differences of behaviour of the liver and kidney enzymes. Further work on the reaction kinetics of these two enzymes and also upon liver and kidney glucuronidases is in progress.

SUMMARY

1. A reinvestigation of the methods for the purification of ox-spleen β-glucuronidase using the variable solvent solubility test has revealed the presence of two fractions having different pH optima. The two enzymes have been separated and purified by fractional precipitation with ammonium sulphate and shown to have the following pH optima: with *l*-menthylglucuronide 4·5 and 5·0; phenylglucuronide 4·5 and 5·2; phenolphthalein glucuronide 4·5 and 5·2.

2. Methods of β-glucuronidase assay using *l*-menthylglucuronide, phenylglucuronide and phenolphthalein glucuronide are presented.

3. The relation of these results to those of previous workers is discussed.

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The Colorimetric Estimation of Stilboestrol, Hexoestrol and their Glucuronides in Urine

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Any attempt to estimate the synthetic oestrogens stilboestrol (4:4′-dihydroxy-$\alpha\beta$-diethylstilbene) and hexoestrol or their detoxication products in urine is complicated by the presence of other phenolic substances which may give rise to interfering colours in procedures depending on colorimetry, or lead to high blank values from control urines. The dual problem presented in such work is to retain a sufficient quantitative recovery of the oestrogen while reducing blank values to a minimum. Sahasrabudhe & Wilder Smith (1947) have largely overcome this problem in the case of dienoestrol by taking advantage of its diene structure and its reactivity with maleic anhydride. For the two more stable oestrogens, stilboestrol and hexoestrol, it appears that no satisfactory method of estimation in the presence of urine has yet been proposed, though attempts have been made to apply the Dingemanse (1940) method to this problem (Dingemanse & Tyslowitz, 1941; Bass & Salter, 1943). The present communication describes methods of preliminary extraction which enable estimations of these two clinically important oestrogens and of their glucuronides to be made in urine by the nitration method proposed by Malpress (1945).

* Part of the work reported in this paper was carried out at the National Institute for Research in Dairying, Shinfield, Reading.

EXPERIMENTAL

Reagents

Ether. Freshly distilled ether (B.P.), giving no more than a faintly positive reaction for peroxide with ferrous ammonium sulphate and potassium thiocyanate, is used at all stages; possible effects of peroxide during hydrolysis are considered later in this paper.

Aluminium oxide. Mayfair brand (Savory and Moore Ltd.) was used with no preliminary treatment.

Calcium hydroxide. Various samples have been used (e.g. Baird and Tatlock labelled 'from marble' or 'low in arsenic'), and there is an indication that oestrogen recoveries may vary slightly from sample to sample. It is advisable therefore to start with sufficient quantities of the purest product obtainable for a programme of work to be completed with one batch, and to carry out preliminary check recoveries of added oestrogens rather than to assume the recoveries found in this study. Coarse particles are removed by sifting through a 100-mesh sieve.

Methods

In developing these methods cow's urine was used. Subsequently simplified modifications suitable for use with human urine were established. The various methods are given separately below and the processes which form the basis of the extractions are summarized in Table 1.

Table 1. *Effect of reagents on free and conjugated stilboestrol and hexoestrol*

	Saturated $NaHCO_3$ solution	Al_2O_3	$Ca(OH)_2$
Free oestrogens	Insoluble	Unadsorbed	Adsorbed
Oestrogen glucuronides	Soluble	Adsorbed	Adsorbed

In all the modifications of the method Al_2O_3 is used for the removal of pigmented and other substances, leading to a reduction of blank values.

Recoveries of oestrogen, adsorbed on $Ca(OH)_2$ from solutions of known strength and estimated after solution in conc. HCl and extraction with ether, indicate that about half the losses associated with the methods may be ascribed to losses at this adsorption stage. This is borne out by a comparison between recoveries from cow and human urines of both the free oestrogens and their glucuronides (see Fig. 1).

Free stilboestrol and hexoestrol in cow's urine

(A) The urine is filtered and made strongly acid to Congo red with conc. HCl. A suitable portion containing 0·5–2 mg. oestrogen is extracted by hand shaking in a separating funnel with 100 ml. ether. Where the probable oestrogen content is not known, 100 ml. urine is a convenient amount for a trial estimation. Emulsions which may form at this stage are broken by centrifugation, or, if separation is nearly complete, by the addition of anhydrous Na_2SO_4. The ether extract is shaken twice with 25 ml. saturated $NaHCO_3$ solution which removes the conjugated (glucuronide) forms of both oestrogens.

(B) The ether extract is now shaken with 50 ml. 2N-NaOH solution which removes the free oestrogen; the alkaline solution after acidifying with 12·5 ml. conc. HCl is back-extracted with 50 ml. ether. The ethereal solution is washed once with 25 ml. saturated $NaHCO_3$ solution and once with 25 ml. water, dried by anhydrous Na_2SO_4 and passed with light suction through an alumina column 5 cm. high prepared in a tube of *c.* 1 cm. diameter. The flask and the column are washed twice with 5 ml. dry ether.

(C) Finely powdered $Ca(OH)_2$ (2 g.) is added to the ethereal solution and, after dispersion through the liquid by careful rotary hand movement for a few seconds, allowed to settle. The ether is decanted and the solid washed once with 15 ml. ether, which is also decanted; these ethereal solutions are discarded. The last traces of ether are evaporated from the $Ca(OH)_2$ by immersing the flask in warm water, the dry solid is suspended in 25 ml. water and dissolved in 7·5 ml. conc. HCl. The acid solution is extracted with 50 ml. ether.

(D) After washing twice with 25 ml. water to free it from acid and drying with anhydrous Na_2SO_4, the ethereal solution is evaporated to small volume and quantitatively transferred to a 50 ml. volumetric flask. It is evaporated to dryness, care being taken to remove any condensate on the neck of the flask by an air current, and the residue dissolved in 6 ml. glacial acetic acid and estimated by the nitration method (Malpress, 1945).

Stilboestrol and hexoestrol monoglucuronides in cow's urine

The saturated $NaHCO_3$ solution from (A) or, alternatively, from a second urine sample treated as in (A), is made strongly acid to Congo red by the addition of 6 ml. conc. HCl and extracted with 50 ml. ether. The ether is dried with anhydrous Na_2SO_4 and, after decanting and washing the solid twice with 5 ml. ether, the combined extract is treated with $Ca(OH)_2$ as in (C) above. The ethereal solution finally resulting from this treatment is shaken twice with 25 ml. saturated $NaHCO_3$ solution and the combined aqueous solutions acidified strongly by the addition of 20 ml. conc. HCl (final conc. 2–2·5N). Hydrolysis is carried out by heating this acid solution under reflux for 0·5 hr.

After cooling, the solution is extracted with 50 ml. ether, and the ether extract washed with 25 ml. saturated $NaHCO_3$ solution, then with 25 ml. water and dried with anhydrous Na_2SO_4. The solution is passed through an alumina column, as for the free oestrogens, and afterwards evaporated to small volume, transferred to a volumetric flask and prepared as outlined above for estimation.

Free stilboestrol and hexoestrol in human urine

The procedure is the same as that for the estimation of free oestrogens in cow's urine except that the adsorption on $Ca(OH)_2$ is omitted. The ethereal solution obtained after passage through the alumina column is immediately concentrated, transferred to a volumetric flask and prepared for estimation.

Stilboestrol and hexoestrol monoglucuronides in human urine

The procedure follows the corresponding method for cow's urine but omits the $Ca(OH)_2$ adsorption. The initial saturated $NaHCO_3$ extracts are acidified with 20 ml. conc. HCl and hydrolyzed for 0·5 hr. Treatment after hydrolysis is the same as for cow's urine.

Estimation

The oestrogen estimations were carried out as described in an earlier paper (Malpress, 1945), except that the stilboestrol solutions were not heated after addition of HNO_3. The final solutions were invariably slightly cloudy and were filtered through sintered glass filters, porosity G4. Filter papers were unsatisfactory, adsorbing appreciable amounts of colour.

Measurements were made with a Spekker photoelectric absorptiometer using a 'spectrum violet' filter (Ilford 601, 430 mμ.) in conjunction with heat-absorbing filters (Hilger H503). Both control and test solutions were read against water in the second cell and with the drum reading at unity.

Hydrolysis

(*a*) Evidence has already been presented (Malpress, 1946) of the partial conversion of stilboestrol to the ψ-(*cis*)-isomer when it is treated with 2·5N aqueous HCl at 100°. The change is accompanied by a loss of 27·5% of the chromogenic power in the nitration reaction. Similar losses are sustained in the hydrolysis of stilboestrol glucuronide, a crystalline sample prepared by the method of Mazur & Shorr (1942) giving only 70% of the theoretical colour development after hydrolysis for 30 min. with 2·5N-HCl.

(*b*) Hydrolysis of the untreated urine results in destruction of added glucuronide either partial (hexoestrol) or complete (stilboestrol). It is essential therefore to prepare extracts as in the methods given before hydrolyzing.

(*c*) Traces of peroxide, if present in the ether used, are carried over to the stage of hydrolysis and greatly reduce recoveries of both oestrogens from simple aqueous solutions. Although this destruction is prevented almost completely by substances normally present in the urinary extracts submitted to hydrolysis, it would seem advisable to avoid all chance of peroxide contamination throughout the process and especially during the hydrolysis (Table 2).

Table 2. *Effect of peroxide on the recovery of oestrogens from their glucuronides after hydrolysis with* 2·5N-HCl

Extracting agent	Recovery (%)	
	Stilboestrol	Hexoestrol
Ether, peroxide free	64	87
Ether, peroxide ++++	0	35
Ether, peroxide ++++ (urinary extract added before hydrolysis)	59	83

(*d*) Complete hydrolysis in 2·5N-HCl is effected in 30 min. with stilboestrol glucuronide, and apparently in about 2 hr. with hexoestrol glucuronide (Table 3). In the latter case there is a progressive destruction of the oestrogen as hydrolysis proceeds for longer periods, indicated by a gradual yellow colouring of the solution, and accompanied by an increasing chromogenic power in the nitration reaction. This being so, it was decided to adopt 0·5 hr. as the time for hydrolysis of hexoestrol glucuronide also, a point at which the hydrolysis is 90% complete and destruction negligible, as shown by the quantitative recoveries of free hexoestrol heated in acid solution with glucurone for this time.

Table 3. *Effect of duration of hydrolysis in* 2·5N-HCl *on the recovery of oestrogens from their glucuronides*

Time (hr.)	Recovery (%)	
	Stilboestrol	Hexoestrol
0·5	71	89
1	71	97
2	69	99
4	—	107
8	—	111

Preparation of glucuronides. Stilboestrol monoglucuronide was isolated from rabbit's urine by the method of Mazur & Shorr (1942). It has been characterized as a dihydrate (Dodgson, Garton & Williams, 1947). Hexoestrol monoglucuronide was similarly obtained, using the ethanol-ether (1 : 3) extraction proposed by Dodgson *et al.* (1947). The sodium salt separates readily from $NaHCO_3$ solution but is pigmented and attempts to remove the pigment by dissolving in water and recrystallizing after the addition of one third volume of saturated $NaHCO_3$ solution were not successful. Recrystallization of the acid from ether, however, gave a purified product (m.p. 179–181°). It is claimed that it is a trihydrate (Dr R. T. Williams, private communication) and the loss of weight on drying at 110° has given figures in accordance with this.

RESULTS

In most cases the individual recoveries given below represent the means of at least four separate determinations. The data on human urines, however, are from duplicates, as are all the figures for hexoestrol glucuronide recoveries. All single measurements fell well within ± 5% of the mean values quoted.

Estimations of the free oestrogens were carried out after the addition of appropriate amounts of a 0·1% (w/v) solution of the oestrogen in glacial acetic acid to 50 or, more usually, 100 ml. of distilled water or urine. The glucuronides were added in faintly alkaline aqueous solution, *c.* 0·1% (w/v) with respect to their oestrogen components, to the same volumes of water or urine. Invariably only single estimations were made on any one sample, the free and conjugated oestrogen recoveries being obtained from separate samples. Where human urine was used results were obtained from both mixed women's and mixed men's specimens, the recoveries being the same in both cases. All results are shown in Fig. 1.

Recovery of free and conjugated stilboestrol (Fig. 1 *a*, *b*). With the process for cow's urine there is a definite increase in the percentage recovery as the original amount of stilboestrol or its glucuronide added to water is increased. Mean recoveries ranged from 66 to 79% for the free oestrogen and from 52 to 64% for the glucuronide (calculated on oestrogen content), as the weight added increased from 0·5 to 2·0 mg. This variability was not found in the mean recoveries from cow's urine which were approximately 70 and 60% respectively, for all

amounts added. The reason for this discrepancy is not clear, but since the percentage recoveries of stilboestrol from water after adsorption on $Ca(OH)_2$ show a similar trend it is probable that it is introduced at this stage of the method, and may be ascribed to slight differences in adsorption characteristics due to the presence of other urinary constituents. Similar explanations may be tentatively adduced for the existence of like relationships in the recoveries of stilboestrol and hexoestrol glucuronides.

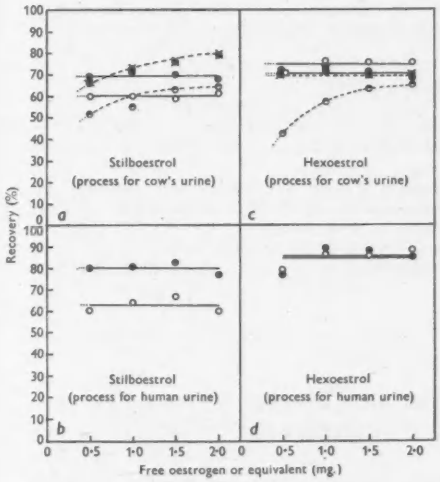

Fig. 1. Recoveries of stilboestrol, hexoestrol and their glucuronides from water and urine. ✕ - - - - - ✕ Free oestrogen from water. ◓ - - - - ◓ Conjugated oestrogen from water. ●——● Free oestrogen from urine (mean value). ○——○ Conjugated oestrogen from urine (mean value).

Recoveries from human urine are higher, and constant mean values of 80 and 63% for the free and conjugated forms, respectively, were obtained over the range studied. The comparatively low recoveries of the glucuronide are of course largely due to the decomposition during hydrolysis, the maximum yield being only 70–75% of the added material.

Recovery of free and conjugated hexoestrol (Fig. 1 *c*, *d*). 70% of the free oestrogen was recovered over the whole range (0·5–2·0 mg.) from both water and cow's urine. The lack of agreement between the recoveries of hexoestrol glucuronide from water and from urine when the method involving lime adsorption is used has already been noted. From urine constant yields of 74% were obtained.

Mean recoveries from human urine over the complete range were equal to 85% for both the free and combined forms.

Blank values from control urines. The methods have been tested for recoveries of 0·5–2·0 mg. oestrogen, and it is suggested that the initial volume of urine for extraction should be chosen to ensure an estimation in this range.

In terms of the chromogenic power of the oestrogens, blank values for the free oestrogen process using cow's urine fell normally within the range 0·05–0·2 mg. oestrogen/100 ml. urine. Occasionally, however, these values were greatly exceeded, blanks of 2 mg. being obtained. Blank measurements for the corresponding conjugated-oestrogen method were, on the other hand, invariably low and again of the order 0·05–0·2 mg. oestrogen/100 ml. urine.

The values given by the simplified modification applicable to human urines have been less than 0·05 mg. oestrogen for the free process, and less than 0·15 mg. oestrogen for the conjugated form, from 100 ml. mixed urine whether from men or women.

DISCUSSION

A preliminary study of oestrogen excretion after the oral administration of a massive dose (5 g.) of stilboestrol to a cow has already been communicated by Malpress & Owen (1947). During a control period satisfactorily constant blank values of 50 and 3 mg. daily were obtained for the free and conjugated extraction processes in terms of oestrogen equivalents and increases in these values greater than 25 and 3 mg. respectively, after administering the oestrogen, could confidently be ascribed to the effects of the treatment.

Excretion studies are to be carried out on human subjects, and it is evident from the blank values to be expected—approximately 0·5 mg. for free and 1·5 mg. for combined oestrogen in a 24 hr. sample—that the sensitivity of the methods should be much greater when thus applied. It seems probable that excretions of the order of 0·5 mg. stilboestrol or hexoestrol daily, whether free or combined, should be readily detectable, and that greater amounts should be estimated with accuracy.

The very low recoveries of conjugated oestrogen reported hitherto from the urines of experimental animals (Stroud, 1939; Dingemanse & Tyslowitz, 1941; Bass & Salter, 1943) have undoubtedly been due to the failure to appreciate the degree of solubility of the glucuronides in organic solvents, and, more especially, the losses entailed when hydrolysis is performed on urine itself, rather than on suitably prepared extracts.

A recent report (Wilder Smith, 1947) claims on indirect evidence an excretion of amounts up to 6%

of the oestrogens administered to human subjects as a sulphate ester. The absence of any detectable change in the excretion of ethereal sulphate after giving the synthetic oestrogens to rabbits (Dodgson *et al.* 1947) and the occurrence of only small changes of doubtful significance in the ethereal S : inorganic S ratio in the cow (Malpress & Owen, 1947) suggest, however, that these substances are normally excreted either in the free form or combined as glucuronide. If this is so the methods described in this paper should be capable of yielding comprehensive data on the quantitative excretion of the oestrogens, and afford some insight into the measure of any metabolic breakdown.

SUMMARY

1. Methods are described for the estimation of the synthetic oestrogens stilboestrol and hexoestrol and their glucuronides in cow's or human urine.

2. After addition to human urine 80% or more of these substances were recovered, except with stilboestrol glucuronide where decomposition during hydrolysis reduces the figure to 63%. Recoveries from cow's urine were slightly lower in all cases.

I wish to express my thanks to Dr R. T. Williams for samples of stilboestrol glucuronide and glucurone, and to Prof. D. C. Harrison and Dr S. J. Folley for their interest in the progress of this work.

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The Fifth Coagulation Factor ('Factor V'). Preparation and Properties

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In 1943 a previously unknown coagulation factor was discovered and termed the fifth coagulation factor, or 'Factor V' (Owren, 1944). Factor V is necessary for the conversion of prothrombin to thrombin, and the rate of this reaction increases with increasing amounts of Factor V up to a certain limit. Lack of Factor V produces a haemorrhagic diathesis, termed *parahaemophilia* (Owren, 1946, 1947*a*). The isolation of this factor and its function in the coagulation process has been discussed in detail elsewhere (Owren, 1944, 1945, 1946, 1947*b*).

In the present paper experiments are described on the basis of which a new method for the preparation of Factor V has been developed, providing a more highly purified product.

METHODS

Ox plasma

Ox blood (9 vol.) was mixed with 1 vol. of 4% (w/v) potassium oxalate monohydrate solution, the mixture cooled to 1° and the plasma obtained by centrifuging at this temperature.

Prothrombin-free plasma

In order to obtain Factor V absolutely free from prothrombin or thrombin, prothrombin-free plasma was prepared. Prothrombin is far more readily adsorbed than Factor V by adsorbents such as $Mg(OH)_2$, $Al(OH)_3$ and $Ca_3(PO_4)_2$, but it is difficult to obtain plasma absolutely free from prothrombin with these agents. On the other hand, 10–40% of Factor V in the plasma is adsorbed simultaneously with the prothrombin depending on the agent used and the relative quantities of adsorbent and plasma (Owren, 1947*b*, p. 78). Asbestos differs from these agents in adsorbing prothrombin selectively. Using asbestos-paper filter pads containing 30–40% asbestos, prothrombin-free plasma with an almost unchanged content of Factor V can be obtained. Filters of higher asbestos content remove some of the Factor V.

The following procedure was adopted. The plasma was passed through a clarifying filter containing about 20% asbestos, and then twice through pads containing 40% asbestos (Hodgkinson, Wookey Hole, Somerset). Before filtration the pads were washed with citrate-saline (0·4% (w/v) trisodium citrate, 0·9% (w/v) NaCl).

Preparation of Factor V

Principle

Human fibrinogen prepared by the ether-fractionation method of Kekwick, Mackay & Record (1946) appeared to be free from Factor V, and this encouraged an examination of the applicability of the procedure to bovine plasma.

The crude fibrinogen precipitate obtained by addition of 0·1 vol. ether to plasma at unadjusted pH and 0° was found to contain 10% of the plasma Factor V. This was removed by repeated washing with distilled water and the fibrinogen was further purified by reprecipitation.

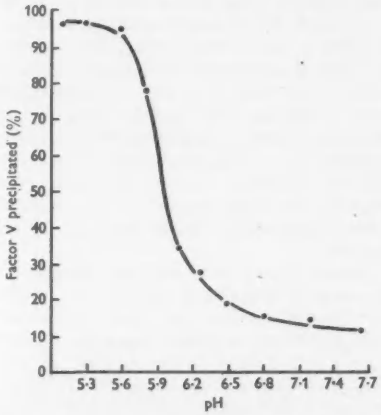

Fig. 1. The precipitation of Factor V from diluted plasma with 0·1 vol. ether at various pH values ($\mu = 0{\cdot}05$, and $-1°$. Protein conc. approx. 2%).

After the removal of the fibrinogen, about 50% of the plasma globulins were precipitated by adjusting the pH to 6·5 and lowering the ionic strength to $\mu = 0{\cdot}05$ by dilution with distilled water, the ether being maintained at 10% (v/v). The supernatant fluid from this precipitation contained substantially all the plasma Factor V.

The pH of samples of this fluid was further lowered and the precipitates which formed were assayed for Factor V. The precipitation of Factor V as a function of pH is shown in Fig. 1. It can be seen that by lowering the pH to 5·3, the Factor V is almost completely precipitated. The amount of protein and Factor V extracted from this precipitate by acetate buffers of pH 5·23 and varying ionic strength is shown in Fig. 2. From this it is obvious that by extracting the precipitate with acetate buffer, pH 5·23, $\mu = 0{\cdot}04$, about 30% of the protein can be removed with only a minimal loss of Factor V.

The precipitate formed at pH 5·3 contains a sticky yellow substance which like Factor V is soluble in water at pH 6·5 and above, but unlike Factor V is insoluble in acetate buffer, pH 5·23, $\mu = 0{\cdot}10$. Consequently it can be removed by dissolving the precipitate and reprecipitating under these conditions. In the presence of a certain amount of sodium chloride the solubility of Factor V is increased, and by this means the loss of Factor V in the precipitate is reduced to about 10%.

On the basis of these findings, the following preparative procedure was developed.

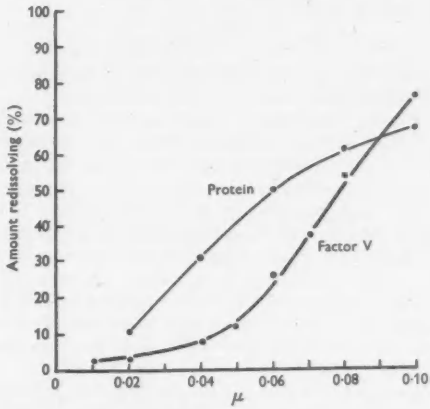

Fig. 2. The solubilities of Factor V and protein (precipitated from plasma at pH 5·3 with ether) in acetate buffer at pH 5·23 and various ionic strengths.

Procedure

(1) Prothrombin-free ox plasma was diluted with 2 vol. of distilled water, cooled to $-1°$, and 10% (v/v) ether was added slowly through a capillary jet, the temperature being kept at -1 to $-2°$. The precipitate was allowed to settle for 3 hr. and was then removed and used for the preparation of fibrinogen.

(2) The supernatant fluid was adjusted to pH 6·5 by the addition of 0·1 N-acetic acid and left for 2 hr. at $-1°$. The precipitate was removed.

(3) The supernatant fluid from (2) was adjusted to pH 5·3 by further addition of 0·1 N-acetic acid. After 1 hr. at $-1°$ the precipitate was collected by centrifuging at $-2°$. This precipitate contains about 80% of Factor V in the plasma.

(4) The precipitate was washed at 0° with acetate buffer, pH 5·23, $\mu = 0{\cdot}04$, by suspending and centrifuging. It was then suspended in a volume of distilled water equal to 20% of the original plasma volume, and dissolved by adjusting the pH to 6·5 with 0·1 N-NaOH. Factor V dissolved completely, and a small amount of undissolved material was removed by centrifuging.

(5) To the solution NaCl was added to give an ionic strength of $\mu = 0{\cdot}10$, and the pH was then lowered to 5·3 by the addition of $\frac{1}{3}$ vol. of acetate buffer, pH 5·23, $\mu = 0{\cdot}10$. A precipitate of yellow protein was formed and removed after 1 hr. by centrifuging in the cold.

(6) Factor V was precipitated from the supernatant fluid by lowering the ionic strength to 0·04 by the addition of 150 ml. distilled water to each 100 ml. solution. After 2 hr. at 0°, the precipitate was collected on the centrifuge, washed with distilled water and dissolved by suspending it in distilled water and adding 0·1 N-NaOH to pH 7·0.

(7) The solution of Factor V was dried from the frozen state.

The yield obtained was about 60% and the purification 100–150 times in terms of activity/mg. of nitrogen. It is essential that the whole procedure should be carried out at low temperature as Factor V is inactivated by ether above 0°, especially at pH 5·3.

Properties of Factor V

The dried preparation of Factor V is a white, amorphous water-soluble protein material.

Stability. The dried product, in sealed bottles, is stable indefinitely. When stored in solution at 0° the activity decreases about 50% in the 1st week. Higher temperatures increase the rate of inactivation. The stability on storage of Factor V in human plasma is of the same order, with a reduction to about 30% of the original activity after 8 days at 0°, and to below 10% in the same time at 10°. The rate of inactivation varies considerably. In ox plasma the stability as a rule is greater. The inactivation by heat, acid and alkali was reported upon previously (Owren, 1947*b*, pp. 85, 90).

Adsorption. As stated above, Factor V is partly adsorbable from plasma with $Mg(OH)_2$, $Al(OH)_3$ and $Ca_3(PO_4)_2$. From purified solutions Factor V may be removed almost completely by these agents, and Seitz-filtration reduces the activity considerably.

Function. The influence of Factor V on thrombin formation has been described previously (Owren, 1944, 1945, 1947*b*, p. 182). Without Factor V no thrombin is formed.

The dried preparation of Factor V described above (0·2 mg.) produced the following effects: (1) The time for complete conversion of prothrombin to thrombin in 1 ml. of a mixture containing prothrombin (10 P.U.), thrombokinase (human brain, optimal amount) and Ca (2·5 mM) was shortened from ∞ to 75 sec. at 37°. (2) The coagulation time for 1 ml. of a mixture containing prothrombin (100 P.U.) thrombokinase (optimal amount), Ca (2·5 mM) and fibrinogen (0·10%) was shortened from ∞ to 16 sec. at 37°. (3) The 'prothrombin time' by Quick's method in a case of parahaemophilia was shortened from 68 to 15 sec. The 'prothrombin time' of human oxalated plasma stored 1 week at 5° was shortened from 38 to 16 sec.

DISCUSSION

Quick (1943, 1946) has postulated the existence of two components of prothrombin, designated prothrombins A and B. Prothrombin A is labile and disappears when plasma is stored, prothrombin B is more stable, is completely adsorbed by $Al(OH)_3$ (which does not remove prothrombin A) and its content is lowered in dicumarol poisoning.

The hypothesis is chiefly based on the fact that the prolonged 'prothrombin time' (Quick, 1943) of stored plasma is shortened by the addition of plasma, previously treated with $Al(OH)_3$ to remove adsorbable material, or plasma from animals poisoned with dicumarol.

Quick's theory has been supported by others (Oneal & Lam, 1945; Zondek & Finkelstein, 1945; Munro, Hart, Munro & Walking, 1945; Munro & Munro, 1947), but questioned by Link (1945), Banfi, Bay & Tanturi (1945); Loomis & Seegers (1947). Loomis & Seegers (1947) believe that the labile component partially inactivated during storage is fibrinogen. They hold the opinion that plasma treated with $Al(OH)_3$ acts by supplying reactive fibrinogen, since the same effect could be produced with stored plasma by the addition of a fibrinogen preparation.

It has been shown previously that Factor V is the most unstable coagulation component in plasma during storage; prothrombin usually remains unchanged for 6 days in plasma stored at 0°, and the reactivity of fibrinogen keeps unaltered for at least 10 days at 0° (Owren, 1947*b*, pp. 84, 143, 271). The prolonged 'prothrombin time' by Quick's method in stored plasma is, therefore, first and foremost due to the inactivation of Factor V. After storage for a long time, reduced prothrombin concentration and decreased activity of fibrinogen may exert some influence. This is confirmed by the following experimental evidence.

Oxalated human plasma stored at 5° for 8 days showed a decrease of Factor V to 10% of the original value, and of prothrombin to 75%. The prothrombin time was reduced from 34 to 15 sec. on addition of Factor V, whilst the addition of prothrombin (20 P.U. free of Factor V) and fibrinogen (0·10%, free of Factor V and profibrin) gave prothrombin times of 30 and 32 sec. respectively.

The prothrombin time of plasma from a patient with parahaemophilia after storage for 3 weeks at 0° was reduced from 110 to 17 sec. on addition of Factor V, whilst fibrinogen and prothrombin, both free from Factor V, were without influence.

The effect of fibrinogen in the experiment of Loomis & Seegers (1947) can be explained by the fact that fibrinogen precipitated from bovine plasma by the ethanol method used can be shown to contain about 10% of the plasma Factor V. Further, it should be mentioned that fibrinogen which contains profibrin will also shorten the clotting time when added to plasma.

Fantl & Nance (1946) have found that prothrombin-free plasma contains a factor which accelerates

the conversion of prothrombin to thrombin, and Ware, Guest & Seegers (1947*a*) have isolated such a factor by fractionation of prothrombin prepared by $Mg(OH)_2$ adsorption. They suggest that this factor is not identical with any of Quick's prothrombins.

There seems to be no doubt that Quick's prothrombin A and Factor V are identical. The factor is concerned with the conversion of prothrombin to thrombin, however, and is not itself a component of prothrombin. The factor is partly adsorbed by $Mg(OH)_2$, and prothrombin prepared by this method always contains some Factor V. (Prothrombin which is absolutely free of Factor V is not converted to thrombin by thrombokinase and Ca alone (Owren, 1944, 1947*b*, p. 186).) Factor V is precipitated by the ammonium sulphate fractionation used by Ware *et al.* (1947*a*) for isolating the accelerator factor from prothrombin preparations. The relatively slight activity of the material isolated (shortening of the conversion time to 3, 7 and 10 min.) indicates that the amount of Factor V is low.

The mode of action described by Ware *et al.* (1947*b*) for their accelerator factor, however, conforms with the previously described action of Factor V (Owren, 1944, 1945, 1947*b*), and there is no reason to believe that they are different substances.

The recorded properties of Factor V and experiments on a quantitative basis point to the fact that prothrombin A, Seegers's accelerator factor and Fantl & Nance's factor are identical with Factor V.

SUMMARY

1. A new method for the preparation of 'Factor V' is described, and its properties are outlined.

2. Experiments are submitted indicating that the prothrombin A of Quick (1943, 1946) and the accelerator factor of Fantl & Nance (1946) and of Ware *et al.* (1947*a*, *b*) are identical with Factor V.

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The Detection of Creatine and Creatinine by Partition Chromatography

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Methods commonly used for the identification and quantitative estimation of creatinine, based on the Jaffé reaction, suffer from the lack of specificity of this reaction. Hunter (1928) has listed 40 compounds which give a positive creatinine reaction with alkaline sodium picrate. The use of 3:5-dinitrobenzoic acid (Benedict & Behre, 1936), although it provides a slightly more specific reaction, introduces the serious complications of colour fading and sensitivity of the coloured product to the presence of other substances. The picric acid method of estimation is reliable, however, if precautions are taken to exclude the interference of other chromogenic substances, or when combined with specific enzymic destruction of creatinine by *Corynebacterium ureafaciens* (Miller & Dubos, 1937; Krebs & Eggleston, 1939).

The estimation of creatine in the presence of creatinine by the picric acid or 3:5-dinitrobenzoic acid methods has the added disadvantage of being

a method of difference. This is emphasized in the identification of small amounts of creatine in the presence of creatinine in circumstances where the Barritt diacetyl reaction for creatine (Eggleton, Elsden & Gough, 1943) cannot be used and the Jaffé test only can be applied, since the detection must necessarily involve a quantitative determination.

The technique of partition chromatography on paper, for the study of amino-acids (Consden, Gordon & Martin, 1944), may be applied to the examination of inorganic and organic urinary constituents, including creatinine (Dent, 1947). The movements of these substances on paper chromatograms are sufficiently specific to permit their recognition. This principle has been utilized in the qualitative identification of creatine and creatinine on the basis of differences in their physicochemical rather than chemical properties, upon which present analytical procedures rely. Creatinine may be detected in biological fluids in amounts down to 1 μg. Further, creatine may be detected in the presence of creatinine without a quantitative analysis.

METHODS

The experimental procedure employed was essentially that of Dent (1946), temperature control throughout experiments being maintained by placing the apparatus in a water thermostat.

For the detection of creatinine, enough of the test solution to contain 5–10 μg. creatinine was pipetted in portions on to paper strips, and the water dried off. The solvent was allowed to travel 25–30 cm. down the strip, the time taken for the solvents collidine and *sec.*-butanol being *c.* 24 hr. After the strip had been dried at 100° for 30 min., creatinine was located by spraying first with 2N-NaOH, then with saturated picric acid from an atomizer. The resulting orange band was generally *c.* 10 mm. in width. In determining R_F values (Consden *et al.* 1944) it was found necessary to mark the position of the band soon after its appearance, since considerable or complete fading of the colour usually took place in sunlight.

For the detection of creatine, the procedure was similar except that the strip was heated at 110° for 3 hr. to convert any creatine present to creatinine. Subsequent spraying of the strip with NaOH and picric acid indicated the presence of a creatine band, detected as creatinine. An alternative method, described by Maw (1947) for the conversion of creatine to creatinine by spraying the strip with mineral acid and heating to dryness at 100°, has not proved entirely satisfactory, owing to the strips becoming brittle and occasionally charred. The direct heating method has the advantage of simplicity, with the desirable minimum of manipulation.

RESULTS

Constancy of the factors influencing the movement of a compound on a chromatogram, listed by Consden *et al.* (1944), was maintained during runs. Comparison of the R_F values of bands obtained from test material was made with those of creatine and creatinine added to the test material. All R_F values quoted represent the average of 6–12 determinations, deviations from mean values being at the most $\pm 5\%$ and generally not more than $\pm 2\%$.

R_F values of creatine and creatinine. In order to obtain a separation between creatine and creatinine, several solvents were tried, *sec.*-butanol and collidine giving satisfactory results (Table 1). Creatine and creatinine showed no movement in *n*-pentanol, whereas in phenol creatinine moved as fast as the solvent itself. In *n*-butanol the R_F value of creatine is too low to be of practical use.

Table 1. *R_F values of creatine and creatinine in various solvents at* 17°

(20 μl. of 0·1% aqueous solution used.)

	Solvent		
Compound	*n*-Butanol	*sec.*-Butanol	Collidine
Creatine	0·04	0·18	0·19
Creatinine	0·20	0·33	0·46

Table 2. *Effect of temperature on R_F values of creatine and creatinine*

(20 μl. of 0·1% aqueous solution used.)

	Solvent: *sec.*-butanol		Solvent: collidine	
Temperature	Creatine	Creatinine	Creatine	Creatinine
17°	0·18	0·33	0·19	0·46
20°	—	—	0·14	—
25°	0·17	0·29	0·11	0·40

A rise in temperature produced a general decrease in R_F values (Table 2). This is in part due to the influence of temperature on the composition of the solvent and water phases, and the consequent distribution of the compounds between the phases. The effect of extraneous salts may be explained similarly. The effect is more marked with collidine as solvent, the composition of the solvent phase changing from 41·7 to 62·8% collidine over the temperature range 10–30°, in contrast to the change for the *sec.*-butanol-water system of 61·7 to 65·0% *sec.*-butanol. Without temperature control, variations of 40% between values have been obtained.

In Table 3 are given R_F values for creatine and creatinine determined in pure solution, in rat urine and in aqueous muscle extracts. Chromatograms of rat urines gave a single band when the strips were simply dried free of solvent, and an additional slower moving band when the strips were heated at 110°. Chromatograms run with added creatine and creatinine showed that the bands obtained from urine alone were attributable to these compounds. The presence of urinary constituents produced distinct effects on the R_F values of the two compounds, the presence of creatinine in large excess, however,

having no effect on the movement of creatine. The solubility of creatine in water is known to be increased by the presence of urinary constituents and sodium salts as a result of salt formation. An effect on the partition coefficient of this substance between organic solvents and water would therefore be expected.

Table 3. *R_F values of creatine and creatinine at 17°*

(20 μl. of solution used.)

	Solvent		
	n-Butanol	*sec.*-Butanol	Collidine
Creatine:			
0·1% Solution	0·04	0·18	0·19
0·025% Solution containing 0·5% creatinine	—	0·18	—
Rat urine (band I)	—	0·17	0·13
0·1% Creatine added to rat urine	*c.* 0	0·16	0·13
5% Aqueous muscle extract	—	0·16	—
Creatinine:			
0·1% Solution	0·20	0·33	0·46
Rat urine (band II)	0·17	0·29	0·57
0·1% Creatinine added to rat urine	0·17	0·28	0·57

The bands obtained with rat urine were considerably broader than those from the pure solutions, and were much less sharply defined at the edges. Apart from the amount of material used, the width of bands is determined by the presence of salts which tend to distort the bands, either by salt formation with the compound being chromatographed, or by the production of local alterations in solvent and water-phase composition on the strip. As might be anticipated from its amphoteric nature, creatine is the more affected in this way.

R_F values have been determined on pure solutions containing 4–100 μg. of creatinine. Over this range the values were not affected by the amount of compound used. Creatine and creatinine were easily detectable in amounts down to 1 μg.

R_F values of other 'Jaffé-positive' substances. The majority of substances other than creatinine giving the Jaffé reaction may be recognized by further chemical tests, but the detection of creatinine at the same time presents numerous difficulties. These are to a large extent overcome when test solutions are examined by partition chromatography. 'Jaffé-positive' substances such as acetone, acetaldehyde, glyoxal, etc., are eliminated by volatilization during the drying of the paper strips. 'Jaffé-positive' inorganic salts, e.g. ferrous sulphate, showed little or no movement in the presence of organic solvents, and were detectable only in relatively large amounts. Pyruvic and acetoacetic acids gave R_F values close to those of creatine, but quite distinct from those of creatinine (Table 4). Since these substances give a *direct* Jaffé reaction, there is little likelihood of confusion with creatine. Pyruvic acid did not give bands when present in amounts much less than 100 μg. The test was more sensitive for acetoacetic acid, but the resulting bands faded rapidly.

Table 4. *R_F values of some 'Jaffé-positive' substances in pure solution at 17°*

(20 μl. of solution used.)

	Solvent	
Compound	*sec.*-Butanol	Collidine
Pyruvic acid (0·5%)	0·18	—
Acetoacetic acid (0·1%)	0·21	—
Glycocyamidine (0·1%)	0·30	0·43 (0·37 at 25°)

Glycocyamine was not converted to glycocyamidine on paper strips after heating for several hours at 110°. Glycocyamidine, however, gave R_F values in collidine and *sec.*-butanol close to those of creatinine. No separation between creatinine and glycocyamidine on chromatograms has been obtained with the solvents used, mixtures of the two compounds giving rise to one broad band. The bands obtained from equivalent amounts of the separate compounds were considerably different in their rates of colour development with alkaline sodium picrate. The creatinine band gave an intense and almost maximal colour within a few seconds. The glycocyamidine band gave little or no colour in this time, and required at least 10 min. for strong colour development. The various alkyl and acyl derivatives of creatine and creatinine have not been tested.

DISCUSSION

In the identification of creatine and creatinine on the basis of partition chromatography, emphasis is transferred from the lack of specificity of the Jaffé reaction to the similarity in chromatographic behaviour of 'Jaffé-positive' substances present in the test material to that of the pure compounds. It has been shown that one compound most likely to interfere with the detection of creatinine is glycocyamidine, although in the amounts used the two compounds have very different rates of colour formation, which might be used to distinguish the major component of a mixture of both compounds.

The R_F values of creatine and creatinine are sufficiently far apart to give clear-cut separation of the two compounds, and to enable creatine, in the presence of its anhydride, to be identified in mixtures in which the creatine content is 1% of the creatinine present or less. The limit of satisfactory detection of either substance by the Jaffé reaction is 1 μg. With

dilute solutions, the initial pipetting of large volumes on to chromatograms may lead to the accumulation of enough salts seriously to disturb band formation and movement. For creatinine detection under these circumstances, preliminary adsorption on to Lloyd's reagent (Behre & Benedict, 1922) is suggested.

The methods described have not been extended beyond the qualitative detection of creatine and creatinine. Preliminary experiments indicate that it is doubtful whether the direct application of the Jaffé reaction to paper chromatograms can be made sufficiently quantitative, owing to the variations obtained in the width of bands and the difficulty of spraying on standard amounts of the reagents. However, the bands of both compounds, in the particular solvents used, are discrete enough for their positions to be calculated from R_F values which have been determined from duplicate experiments, the bands cut out of the strips and the creatinine extracted from the paper and estimated photocolorimetrically. This principle has been applied with success by Flood, Hirst & Jones (1947) in the chromatographic identification and estimation of sugars.

SUMMARY

1. A method is described for the identification of creatine and creatinine in biological material in amounts down to 1 μg.

2. Creatine may be detected in the presence of large amounts of creatinine without resort to a quantitative estimation.

3. The effects of various factors influencing the reproducibility of R_F values are discussed.

The author wishes to thank Dr H. King for the gift of a sample of glycocyamidine, Dr E. Work for helpful advice and Miss E. Large for technical assistance.

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The Fate of Ingested Creatinine in the Rat

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The published observations on the excretion of ingested creatinine point to a large and unaccounted loss of this substance when it is administered to rat, dog and man, generally not more than 80% of a given dose being excreted in the urine (Hunter, 1928; Wang, 1939; Beard, 1943). Since there appear to be no concomitant changes in blood urea (Folin & Denis, 1912) or in the output of urinary urea and ammonia (Rose & Dimmitt, 1916), the fate of the unrecovered fraction of the dose has been the subject of some speculation. Claims have been made that creatinine retention takes place, or that conversion to creatine occurs (Beard & Jacob, 1939; Beard, 1943). That losses might result from bacterial decomposition in the gut has been suggested (Beard, 1943). In the earlier studies there is no evidence that intestinal absorption of an oral dose was complete and the likelihood of loss of part of a dose by excretion in the faeces does not seem to have been considered. Dominguez & Pomerene (1945) found that in a human subject with an ileal fistula, 75% of an oral dose was recovered from the urine, in agreement with other published data on man, but, in addition, that intestinal absorption was incomplete, creatinine being recovered in the material from the fistula to the extent of 25%, sufficient to account for the apparent 'loss' of creatinine referred to by previous workers.

A study has therefore been made of creatinine absorption in the rat in order to determine whether partial recovery of ingested doses may again be explained on the basis of incomplete intestinal absorption. A series of *in vitro* experiments has also been carried out to examine the possibility of bacterial destruction of creatinine in the gut.

METHODS

Biological. Adult albino rats of the Wistar strain, ranging in weight from 200 to 300 g., were used. Several of the animals were caecectomized 10 weeks prior to the experiment. The rats were housed in pairs in metabolism cages with unrestricted access to powdered rat cake and water. Urine and faeces were both collected under toluene over 24 hr. periods. The type of urine-faeces separator employed (supplied by Laboratory Glass Blowers, 63 Lowlands Road, Harrow, Middlesex), which is in general use in these laboratories, is shown in Fig. 1. The upper flared end is supported in contact with the base of a 10 in. diam. glass funnel. Faeces fall through into a collecting flask and urine runs down the funnel into the circular trough in the

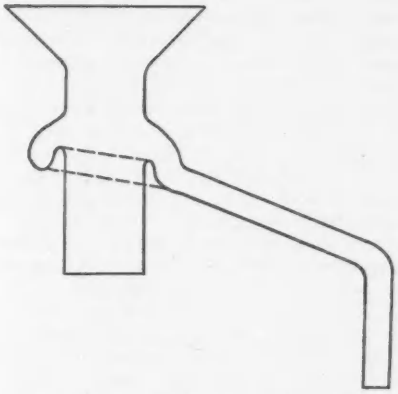

Fig. 1. Urine-faeces separator (side view).

separator, whence it is drained off through the side arm. At the end of each 24 hr. period each collecting funnel and separator was rinsed with water and N-H_2SO_4. For the determination of creatinine alone, the urine and rinsings were made up to 500 ml. and filtered. When creatine and creatinine were being determined, the urine and rinsings were made up to 50 ml. and filtered. The filtrate was diluted 10 times for estimations of preformed creatinine, and the undiluted filtrate used for total creatine + creatinine determinations, the analytical procedure employed giving a final tenfold dilution. The 24 hr. collections of faeces were freed from excess toluene by exposing them to the air on filter papers for 15 min. They were then crushed to a thin smooth paste with a little water and the volume made up to 250 ml. The suspension was shaken for 5 min. and allowed to stand for 15 min. Portions of the supernatant fluid were centrifuged at 5000 r.p.m. for 30 min. and used for creatinine determinations.

Chemical. Creatinine was determined on 2 ml. samples of diluted urine filtrates, and faecal extracts and creatine determined on 5 ml. samples of urine filtrates by methods described by Maw (1947). 'Blank' determinations were simultaneously carried out to compensate for the colour of the faecal extracts and for the slight increase in pigmentation of urines occurring during the estimation of creatine. Creatinine in faecal extracts was estimated enzymically by the method of Miller, Allinson & Baker (1939) for creatinine in blood and urine. The suspensions of creatinine-assimilating *Corynebacterium ureafaciens* were prepared according to the procedure of Dubos & Miller (1937), the bacteria, obtained from soil samples, being subcultured for 10 weeks on a nutrient solution containing 0·5% creatinine. Creatinine present in faecal extracts was identified chromatographically on paper strips with water-saturated collidine and *sec.*-butanol as solvents (Maw, 1948).

RESULTS

Oral administration of creatinine to normal and caecectomized rats. The validity of a study of the fate of ingested creatinine depends on (*a*) accurate measurement of both urinary creatinine excreted in excess of normal amounts and creatinine excreted in the faeces in excess of substances normally present

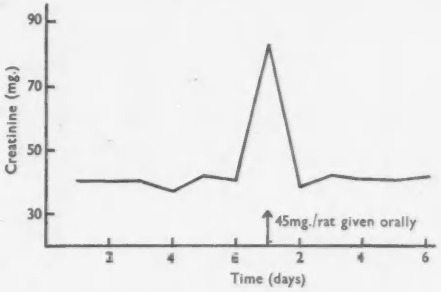

Fig. 2. 24 hr. urinary-creatinine excretion of pairs of rats given creatinine orally.

giving the Jaffé reaction, and (*b*) avoidance of contamination of faeces by urine. To ensure (*a*), pairs of rats were used and were given a preliminary period of 10–14 days in the metabolism cages to accustom them to the experimental conditions. At the end of this period outputs of urine and faeces had become quite steady. Urine and faeces were then collected every 24 hr. for the next 6 days, the dose of creatinine given by stomach tube in 1·5 ml. water, and the urine and faeces collected daily for a further 6 days.

A typical example of urinary creatinine excretion, before and after the oral administration of creatinine to pairs of rats, is shown in Fig. 2. During the first 6-day control period the 24 hr. outputs showed relatively little variation (± 5–7%), urinary creatinine rising immediately after administration of the dose and returning to predosage levels by the next 24 hr. period. In order to minimize the effect of a possible variation in normal output on the day of administration, the difference between the total creatinine outputs for the first and second 6-day periods was taken as being due to the oral dose.

The excretion of substances in the faeces giving the Jaffé test (termed faecal $J+$ material) showed a similar pattern over the experimental period. The 24 hr. outputs from different pairs of rats, expressed as creatinine, were 2–5 mg./rat, and for a given pair of rats showed a maximum variation of $\pm 17\%$ from mean values. Forty analyses of faeces from one pair of rats gave values of $J+$ material, expressed as creatinine, ranging from 0·20 to 0·26 mg./g. fresh faeces.

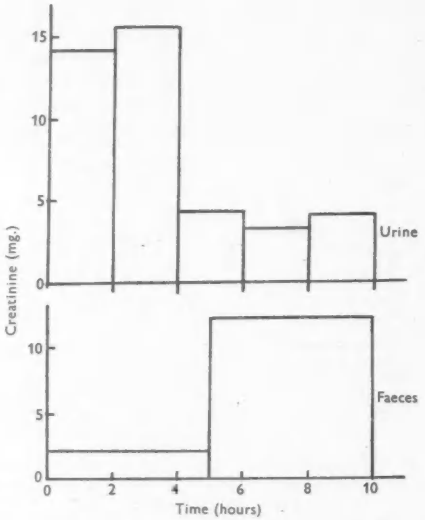

Fig. 3. Urinary and faecal excretion of creatinine in two caecectomized rats following an oral dose (25 mg./rat).

The possibility of contamination of faeces by urine was checked. The type of urine-faeces separator used in these experiments was very reliable in preventing such contamination. Sixty-seven routine analyses of faeces collected from the metabolism cages gave values for $J+$ material/g. agreeing with those obtained from faeces collected directly from rats during defaecation. This was verified under experimental conditions by following the course of excretion of creatinine in pairs of rats for 10 hr. immediately following its oral administration. A typical result for two caecectomized rats is given in Fig. 3. Changes in faecal $J+$ material occurred independently of changes in the level of urinary creatinine. The peak urinary excretion was reached within the first 4 hr., and had fallen to predosage levels after 6 hr. The increases in faecal $J+$ material occurred only within the second 5 hr. period, and during the first 5 hr., when contamination by urinary creatinine would have been most likely, the amount/g. faeces remained the same as that of faeces collected with precautions against any contact with urine. No noticeable effect on excretory levels was produced by control doses of water.

The $J+$ material normally present in rat faeces has not been identified, but the rise in faecal $J+$ substances resulting from an oral dose of creatinine appears to have been due to this compound. This seems certain for the following reasons:

(i) *Behaviour of the faecal 'creatinine' in the presence of creatinine-destroying enzyme suspensions from* Corynebacterium ureafaciens. The use of the enzymic method directly on faecal suspensions or extracts was not wholly satisfactory on account of the presence of enzyme-inhibiting substances in rat faeces. Creatinine solutions which were destroyed to the extent of 95% by the enzyme preparation were only destroyed to the extent of 42–49% in the same time when faeces were present. When, however, the faecal 'creatinine' at equivalent concentrations was incubated with the enzyme suspension, it was assimilated to the same extent.

(ii) *Identification of the faecal 'creatinine' by partition chromatography.* R_F values of creatinine added to faeces were compared with those of faecal 'creatinine' using one-dimensional chromatograms. The values obtained for solvents collidine and *sec.*-butanol are shown in Table 1.

It has been pointed out (Maw, 1948) that of other Jaffé-positive substances, at least one, glycocyamidine, has R_F values close to those of creatinine. However, from the rate of colour development in the presence of alkaline sodium picrate there is strong indication that the substance present in the faeces is creatinine. The fact that the amount of faecal $J+$ material in excess of the normal output, when expressed as creatinine, together with the extra creatinine excreted in the urine accounted in every

Table 1. *R_F values of creatinine and faecal 'creatinine' at 17°*

Dose of creatinine (mg./rat)	Solvent: collidine		Solvent: *sec.*-butanol	
	Faecal 'creatinine'	Creatinine added to faeces	Faecal 'creatinine'	Creatinine added to faeces
45	0·45	0·46	0·26	0·27
45	0·46	0·46	0·27	0·28
45	0·44	0·45	—	—
60	0·45	0·46	0·27	0·27
60	0·44	0·46	0·27	0·27

case for the administered dose (as described below), is taken as additional support.

Creatinine was fed to pairs of rats in doses ranging from 30 to 60 mg./rat in a further series of 12-day experiments. Both normal and caecectomized rats were used to check the possibility of destruction of creatinine by intestinal bacteria as discussed in the note below. In both these and the 10 hr. experiments part of the dose appeared in the faeces. The short-term experiments indicated that this was the result of incomplete intestinal absorption, since the increase in $J+$ material in the faecal pellets coincided with the appearance of animal charcoal given simultaneously with the dose. The results for the 12-day experiments are given in Table 2. The recovery from the urine varied considerably, ranging from 40 to 85 % of the dose. This agrees with other published data. The faecal creatinine excreted amounted to 10–44 %, and together with the fraction excreted in the urine gave almost complete recovery (91–101 %) of the administered dose in every case. In one experiment, urinary creatine was also determined for periods of 11 days before and after administration. The total creatine outputs for the two periods were 121·9 and 114·8 mg./pair of rats. No extra creatinuria took place as a result of the creatinine administration. Daily variations from the mean 24 hr. output were much larger than in the case of creatinine, being *c.* ± 50 %. A dose of creatinine given by injection produced no rise in faecal $J+$ material, indicating no apparent excretion into the intestinal tract; 99 % of the dose was recovered in the urine.

Note on the destruction of creatinine by intestinal bacteria. Twort & Mellanby (1912) reported that several commonly occurring organisms were capable of metabolizing creatine, including a large Gram-positive anaerobic bacillus present in the intestine of the cat from the duodenum to the rectum, and a Gram-negative colon bacillus present in human faeces. They pointed out that the possibility of 'retention' of creatine in ingestion experiments being partly due to bacterial action could not be excluded. This might also apply in the case of creatinine administration, although the recovery experiments with normal rats and with caecectomized rats, in which the time taken for material to pass through the gut was reduced from about 12 to 8–9 hr., indicated little or no loss by this path. In a series of *in vitro* experiments, creatinine solutions were incubated aerobically and anaerobically with crushed fresh faeces from rats under a variety of conditions to determine the extent of bacterial destruction. Under the conditions found most favourable for creatinine breakdown, there was no aerobic destruction within the first 10 hr. Anaerobically, creatinine was found to be stable in the presence of faeces for 5–7 hr., destruction being detectable after this time and practically complete after 20–30 hr. The destruction in the presence of complete intestinal contents of rats under conditions most favourable for bacterial action was very much slower, *c.* 25 % in 20–25 hr. The period during which creatinine was found not to be destroyed in the presence of faeces is of the same order as the time it would be present in the gut of a rat. Although no more than a qualitative comparison may be drawn between the *in vivo* and *in vitro* experiments, the latter suggest that any breakdown occurring during ingestion experiments is not extensive, as borne out by the recovery values.

Table 2. *Urinary and faecal excretion of creatinine in pairs of rats after an orally administered dose*

(*n* = normal; *c* = caecectomized; * = given by subcutaneous injection.)

Dose (mg./rat)	Rat	Amount of dose (%) in Faeces	Amount of dose (%) in Urine	Amount of dose recovered (%)
40	*c* ♂	—	40·9	—
45	*n* ♀	—	48·9	—
60	*c* ♂	—	47·5	—
30	*c* ♂	44·0	47·2	91·2
30	*n* ♂	28·3	70·2	98·5
45	*n* ♂	10·1	85·4	95·5
60	*c* ♂	34·4	67·0	101·4
45*	*n* ♂	0·0	98·7	98·7

DISCUSSION

The conversion of creatinine to creatine *in vivo* has been claimed on the grounds that creatinine ingestion or injection have been shown to give rise to temporary increases in muscle creatine in rabbits (Myers & Fine, 1913), rats (Beard & Jacob, 1939) and chicks (Almquist, Mecchi & Kratzer, 1941), with occasionally an accompanying creatinuria (Towles & Voegtlin, 1912; Beard & Jacob, 1939). However, none of the studies in which the creatinine was given orally included an examination of faeces for the compound, and no proof was provided that intestinal absorption was complete. That ingested creatinine was only partly absorbed from the small intestine of the human subject of Dominguez & Pomerene (1945) suggests that it is unnecessary to postulate any metabolic transformation of creatinine to account for the total quantity administered.

Furthermore, the creatinine-creatine conversion could not be verified by Bloch & Schoenheimer (1939) using ^{15}N-labelled creatinine fed to rats.

The findings in this study on the oral administration of creatinine to rats have confirmed those of Dominguez & Pomerene (1945) on a human subject. The increased urinary excretion amounted to as much as 85% of the dose. Creatinine was also found in the faeces, and appeared along with simultaneously administered animal charcoal. The practically quantitative recovery of administered amounts from urine and faeces together suggests that creatinine suffers little or no destruction in the body or intestine, and that the fraction of the dose which is absorbed is quantitatively excreted by the kidney.

The excretion of creatine was followed for two consecutive 11-day periods before and after creatinine administration. No increased creatinuria was observed. Beard & Jacob (1939) claim to have obtained a rise in muscle creatine of rats of 14–41%, together with pronounced increases in creatine excretion. Beard (1943) has stated: 'under normal conditions...it is very easy to show a new production and excretion of excess creatine from its various precursors in the diet'. Yet in the same chapter in this monograph appears the statement: 'wide variations in creatine and creatinine excretion may and usually do occur in a given individual or in different individuals'. The latter is the general finding, and deprives the former statement of its validity. The daily output of creatine is subject to considerable fluctuation, making a creatinuria due to experimental causes difficult to evaluate with any precision over short periods of time.

The fate of ingested creatinine can be adequately accounted for on the basis of its partial absorption and immediate and complete excretion, without introducing the assumption that part of a dose is retained, hydrated or otherwise metabolized. Until further information is available, another explanation must be sought for the changes in muscle creatine which have been reported. Injected creatinine has been shown to undergo complete excretion via the kidney only. This is in agreement with the studies of Dominguez, Goldblatt & Pomerene (1935) on dogs, and those of Dominguez & Pomerene (1945) on humans, in contrast to the findings of Beard (1943). This result is in accordance with the ingestion experiments, again indicating that creatinine entering the blood stream is entirely eliminated.

Qualitatively, administered creatine behaves similarly. Only a small fraction of a dose is excreted in the urine and no apparent changes in urinary urea and ammonia are produced. Some creatine enters the muscles, but not in sufficient amounts to account for the whole of the unrecovered portion. Intestinal breakdown by bacteria has been cited as probable. From the above observations on ingested creatinine it is thought that the low urinary recovery of creatine may also be due, in part at least, to its incomplete absorption.

SUMMARY

1. Creatinine administered orally to normal and to caecectomized adult rats in doses of 30–60 mg./rat was excreted in the urine in amounts up to 85% of the dose. No increased creatinuria was induced.

2. Creatinine also appeared in the faeces, the fraction of the dose excreted in this manner together with the fraction eliminated in the urine accounting completely, within the limits of experimental error, for the administered dose.

3. Orally administered creatinine is only partly absorbed from the intestine, the portion of the dose which is absorbed being immediately and completely excreted. It is considered unnecessary to postulate any metabolism or retention of orally administered creatinine.

4. In experiments *in vivo* and *in vitro*, creatinine has been found to suffer little or no bacterial destruction in the intestine.

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The Effect of Fertilizers on the Levels of Nitrogen, Phosphorus, Protease, and Pectase in Healthy Tobacco Leaves

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Boiled or dried leaves have generally been used in obtaining the data already in the literature on the effect of fertilizer treatment on nitrogen and phosphorus levels in tobacco leaves (Vickery, Pucher, Wakeman & Leavenworth, 1940; Ward, 1942). In addition, much work has been done on cured material.

In the work reported in this paper methods of fractionation were such that sap and fibre fractions were obtained from fresh material. Nitrogen and phosphorus determinations were made on all fractions, and, in addition, the levels of protease and pectase were determined on some.

METHODS

Growing of the plants. Healthy tobacco (*Nicotiana tabacum* var. White Burley) was grown in pots in a heated glasshouse by the Plant Pathology Department. Potting material (1 kg./pot) was made up of 50% soil, 25% sand and 25% peat. Analyses of the mixture indicated that it was relatively deficient in N and P, but not lacking in K. Supplements of fertilizer, when added either singly or in combination, were at the rate of 2·8 g. $(NH_4)_2SO_4$, 0·75 g. $Ca(H_2PO_4)_2$ and 1·1 g. K_2SO_4/pot. The fertilizer was mixed with the soil when the plants were potted. In each experiment eight groups of five pots were used. The fertilizer treatment in the eight groups was: nil; N; P; N and P; K; N and K; P and K; N, P and K. Table 1 gives the cultural history of the plants and the method of sampling employed.

Harvesting of the plants. Usually the plants of half the fertilizer groups were harvested 3 days later than the others (the former having been used as controls for similar fertilizer treatments with virus-infected plants to be described in a subsequent paper). Plants were cut at soil level, adhering soil brushed off, and each plant was weighed to the nearest 0·1 g. In some experiments all the leaves were removed (Table 1), while in the others five leaves of corresponding position and age were removed from each plant. Finally the five lots of leaves in the group were pooled and weighed.

Fractionation. Within 1 hr. of harvesting the samples from each treatment were minced in a domestic meat mincer and the sap expressed by hand through madapollam. The residue after hand squeezing was reminced and again squeezed. The liquid obtained was termed 'crude sap' and the residue of fibrous material 'unwashed fibre'. Washings from the mincer were added to the unwashed fibre, and more water added to bring the total to about five times the volume of crude sap. This fibre suspension was squeezed through the cloth, and the fluid obtained called 'washings'. Washings and crude sap were kept separate, the volumes taken, and the fibre (washed fibre) weighed.

Crude sap was spun on a centrifuge for 15 min. at 3500 r.p.m. ($1500 \times g$), and the supernatant fluid decanted from the deposit of cell debris, starch and chloroplastic material. The supernatant fluid was called 'sap' and the deposit 'sap sediment'.

Analyses. (1) *Dry matter.* Measured portions of each fraction were dried overnight at 95–100° and weighed.

(2) *Nitrogen.* Total N was determined by a micro-Kjeldahl method using SeO_2-$CuSO_4$-K_2SO_4 (1 : 1 : 8) catalyst. Non-protein N was determined by analysis of the supernatant fluid obtained after the addition of an equal volume of 10% trichloroacetic acid. Non-protein N estimations were made on the washings in the first experiment, but not subsequently, as the results obtained showed that the washings were equivalent to diluted sap. For the purposes of calculation it was assumed that errors introduced by treating sap sediment and fibre N as protein N would not be large (Granick, 1938).

Table 1. *Cultural history of the tobacco plants*

Date potted	Date harvested	Sunshine (hr.)	Average weight of whole plant with full fertilizer supplement (g.)	Method* of sampling (leaves taken)
(1) 11. x. 46	30. xii. 46–2. i. 47	158–167	34·1	5
(2) 31. x. 45	21. ii. 46–26. ii. 46	196–222	92·0	5
(3) 4. ii. 46	26. iv. 46–2. v. 46	187–195	105·2	5
(4) 21. v. 46	11. vii. 46–16. vii. 46	290–333	144·5	All
(5) 31. vii. 46	23. ix. 46–26. ix. 46	228–238	88·0	All

* Five largest leaves on 17. xii. 46, 11. ii. 46 and 16. iv. 46, respectively in (1), (2) and (3) were marked, and harvested on the dates shown in the table.

(3) *Phosphorus* was determined by a modification of the method of Kuttner & Lichtenstein (1932).

(4) *Pectase* was determined on sap and on washed fibre (Na_2HPO_4 extract, pH 8) by the method previously described (Holden, 1946).

(5) *Protease* was determined on sap and washed fibre by the method previously described (Tracey, 1948).

Analysis of data. The data obtained were analyzed statistically by the methods described by Yates (1937). The results of this analysis are given in the form of main effects of N, P and K, and the interaction of N and P, other interactions being negligible. The treatment effect is one quarter of the difference between the sum of the values of the four sets receiving a treatment and the sum of the values of the four not receiving it, e.g. N effect = ¼(N + N and P + N and K + N, P and K) − ¼(O + P + P and K + K). Thus if half the treatment effect is added or subtracted, according to sign, to or from the mean of all the treatments, the value for the leaves with that supplement is obtained. The standard errors given are for the treatment effects. Treatment effects greater than twice their standard error are regarded as significant. Extreme ranges and mean values of all treatments combined, and the means for no treatment group and N, P and K group separately, are tabulated. In all, 200 plants were harvested giving 40 results for groups of five plants. Figures for the two methods of sampling have not been separated. This affects the values quoted for total dry matter, N and P, but does not alter the significance of the fertilizer effect on these values.

RESULTS

Dry matter, nitrogen and phosphorus. The results obtained are given in Tables 2–4.

Protease. The results are given in Table 5. For the purpose of calculating the total protease of the leaves, it was assumed that the activity/g. protein N of the washings was the same as that of the sap, and that the sap sediment fraction had no activity. The figures for total protease/g. total protein N indicate

Table 2. *Variation in dry-matter content of tobacco plants*

	Extreme range	Mean of all treatments	Mean of untreated group	Mean of N, P and K group	Fertilizer effects: N	P	K	N and P	Standard error
Wet weight of whole plants (g.)	35·2–722·4	204·49	99·8	445·4	+98	+185	+35	+84	±21·9
Dry matter (% of wet weight of leaves)	7·1–16·8	11·9	11·7	12·2	−0·9	+ 1·3	+0·1	+0·2	± 0·33
Total dry matter (g.)	1·80–64·50	17·22	10·12	31·85	+6·37	+12·85	+1·22	+6·06	± 2·21
Dry matter (mg./ml. sap)	31·2–71·1	50·3	50·3	54·1	+3·0	+ 0·05	−0·6	+3·5	± 1·3
Fibre dry matter (% total dry matter)	42–65	51·2	49·6	51·4	−2·9	+ 4·8	+0·5	−0·2	± 1·32

Table 3. *Variation in nitrogen content of tobacco plants*

	Extreme range	Mean of all treatments	Mean of untreated group	Mean of N, P and K group	Fertilizer effects: N	P	K	N and P	Standard error
Total N (% of dry matter of leaves)	1·03–6·89	3·25	2·85	3·22	+1·90	− 0·94	−0·35	− 0·22	± 0·22
Total N (mg.)	75–2048	475	244·6	821·6	+425	+203	−45	+165	±66
N (mg./ml. sap)	0·94–4·52	2·20	1·86	2·23	+1·42	− 0·82	−0·22	− 0·34	± 0·51
Protein N (mg./ml. sap)	0·47–2·62	1·20	1·04	1·56	+0·63	− 0·08	−0·03	+ 0·16	± 0·16
Sap N (% of sap dry matter)	1·84–9·23	4·41	3·61	4·29	+2·72	− 1·60	−0·31	− 0·88	± 0·24
'Sap sediment' N (% of non-fibre N)	9·9–30·3	17·9	17·0	15·2	−3·0	+ 0·3	−0·5	+ 1·1*	± 1·23
Sap protein N (% of sap N)	33–82	57·2	57·6	69·2	−4·6	+11·8	+2·3	+11·1	± 1·95
Fibre N (% of total N)	29–59	42·1	42·4	45·0	−3·8	+ 9·2	+0·9	+ 3·3	± 1·55
Fibre N (% of fibre dry matter)	0·74–5·65	2·65	2·41	2·77	+1·35	− 0·37	−0·33	+ 0·17	± 0·19

* Appears to be a negative P and K effect of 2·9.

Table 4. *Variation in phosphorus content of tobacco plants*

	Extreme range	Mean of all treatments	Mean of untreated group	Mean of N, P and K group	Fertilizer effects N	P	K	N and P	Standard error
Total P (% dry matter)	0·09–0·52	0·245	0·14	0·34	+ 0·015	+ 0·204	−0·022	+ 0·060	±0·018
Total P (mg.)	2·5–187·2	42·9	14·1	89·1	+18·8	+55·3	−1·8	+18·0	±8·2
Sap P (% sap dry matter)	0·09–1·09	0·331	0·14	0·47	− 0·046	+ 0·361	−0·045	− 0·049	±0·041
Fibre P (% total P)	26–58	42·1	48	39	+ 3·0	− 6·5	−3·6	+ 4·4	±1·95
Fibre P (% fibre dry matter)	0·08–0·40	0·189	0·14	0·23	+ 0·039	+ 0·112	+0·043	+ 0·026	±0·0124

Table 5. *Variation in protease content of tobacco plants*

	Extreme range	Mean of all treatments	Mean of untreated group	Mean of N, P and K group	Fertilizer effects N	P	K	N and P	Standard error
Total protease (units/g. dry matter)	0·6–8·9	3·63	2·90	4·46	−0·30	+1·09	+0·36	+0·28	± 0·30
Protease (units/g. total protein N)	20–428	157	126·8	172	−86	+71	+25	+45	±18·9
Protease (units/ml. sap)	0·03–0·63	0·30	0·23	0·37	−0·097	+0·093	+0·059	−0·013	± 0·031
Protease (units/g. protein N in sap)	25–1040	310	209	255	−261	+124	+86	−125	±51
Protease (% in sap)	24–86	59	57·4	56·6	−8·4	−3·0	+3·1	−5·5	± 3·7
Protease (units/g. dry fibre)	0·8–12·4	3·00	2·34	4·18	+0·74	+1·12	−0·05	+0·72	± 0·42
Protease (units/g. fibre N)	22–262	117·5	98·2	145·8	−38	+57	+15	+0·9	±11·7

Table 6. *Variation in pectase content of tobacco plants*

	Extreme range	Mean of all treatments	Mean of untreated group	Mean of N, P and K group	Fertilizer effects N	P	K	N and P	Standard error
Fibre pectase (units/g. dry matter)	0·066–1·31	0·35	0·40	0·23	+0·17	−0·35	−0·01	−0·07	±0·06
Fibre pectase (units/g. N)	4·4–33·4	12·6	17·5	8·4	−1·1	−8·2	+1·3	+0·8	±1·7

the relative richness of the leaves in protease, and show that if all the leaf protein were hydrolyzed at the same rate as gelatin it would be completely destroyed by the leaf protease in 2–10 days (depending on the fertilizer treatment) at 40°.

Pectase. The effect of fertilizer treatment on the pectase content of tobacco leaves was mentioned briefly in a previous paper (Holden, 1946). In the present series of observations pectase was determined in sap and fibre. The results (Table 6) given do not include those for sap, because in every instance the values were low, and not more than 10% of the total pectase was present in the sap. Thus the earlier observation that the application of N and P together increased the percentage of sap-soluble enzyme was not confirmed. The fibre pectase is expressed as units/g. dry matter and units/g. N of fibre (Holden, 1946). The results of one experiment are not included as several values were missing.

DISCUSSION

The effects found are summarized in Table 7. In spite of seasonal variations in the size and composition of the plants it was found that significant fertilizer effects were obtained from the pooled data of all experiments. While the seasonal effects are of undoubted importance it was felt that the results obtained were not sufficient for discussion in this paper.

Table 7. *Summary of effects of fertilizers on tobacco plants*

Character	Change
Wet weight Total dry matter Total N Total P Fibre P as % fibre dry matter	Increased by both N and P
Dry matter as % wet weight Fibre dry matter as % total dry matter Fibre N as % of total N Protein N as % of total sap N Protease/g. protein N of sap Protease/ml. sap Protease/g. total protein N Protease/g. fibre N	Decreased by N Increased by P
Total N as % dry matter Sap N as % dry matter of sap Pectase/g. dry matter of fibre	Increased by N Decreased by P
Sap dry matter/ml. Fibre N as % dry matter of fibre Total N/ml. sap Protein N/ml. sap	Increased by N
Total P as % dry matter Sap P as % dry matter of sap Total protease/g. dry matter Protease/g. dry matter of fibre	Increased by P
Pectase/g. N of fibre Fibre as % total P	Decreased by P
'Sap sediment' N as % non-fibre N Sap protease as % total protease	Decreased by N

The obvious effects of both N and P supplements in making the plant grow to a greater total size explain their joint action in increasing wet weight, total N, and total P. In respect of many effects, N and P appear to act in opposition. This will be seen by inspection of the second section of Table 7 to be due to P increasing the proportion of fibre, mainly carbohydrate in nature, while N increases the proportion of protein and other nitrogenous compounds. The proportion of the total N that is soluble is increased by N as fertilizer, and similarly P increases the proportion of total P that is soluble. These results might be expected. The effects of N and P on enzyme levels are, however, of more interest. The increase of protein due to N seems to be coupled with a decrease in the enzyme responsible for its breakdown, while the increase of 'fibre' (presumably involving an increase in pectin) due to P is coupled with a decrease in the enzyme responsible for beginning the breakdown of part of the fibre. Unfortunately, the rigid interpretation of the results of experiments such as these is difficult, since the two fertilizers N and P affect the levels of all quantities measured. Consequently there is no constituent to which changes can be referred. Total dry matter and total protein N have been used in the calculation of protease levels. As the total dry matter may contain from 6 to 40 % of its weight as nitrogenous compounds (if the conventional factor of 5·8 is used for conversion) and the protein level is obviously influenced by N as fertilizer, comparison of effects is difficult. The difficulty may be put in another way: both protein and carbohydrate may be laid down in excess of the normal requirements (whatever these are), and there is no way of determining the size of these 'stores' or, alternatively, of deciding whether too little of these compounds is present for 'health'. The amount of a substance present in a plant may be regarded as a reflexion of the balance between its synthesis and breakdown. The changes in enzyme levels described are compatible with the view that these enzymes, which have a destructive action *in vitro*, are not capable of synthesis *in vivo*, by reversal of their action, and that other synthetic systems are involved.

No significant effects of potassium were observed, presumably because the K content of the soil was such that plants without K supplement were not deficient.

SUMMARY

1. The effect of supplements of nitrogen and phosphorus on the nitrogen and phosphorus content of leaf fractions of healthy pot-grown tobacco has been determined. Experiments were carried out in five sets, each including eight different fertilizer treatments on groups of five plants.

2. The effect of nitrogen and phosphorus on the levels of two enzymes, pectase and protease, was also determined on the same material.

3. Phosphorus increases the non-protein, or carbohydrate, components of the leaf, while nitrogen increases the protein components.

4. Nitrogen increases pectase levels, while phosphorus decreases them. The reverse is true for protease. It is suggested that this may indicate the presence of different paths for the synthesis and breakdown of both pectin and protein.

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The Effect of Infection with Tobacco-Mosaic Virus on the Levels of Nitrogen, Phosphorus, Protease, and Pectase in Tobacco Leaves and on their Response to Fertilizers

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The effect of fertilizers on the levels of nitrogen, phosphorus, protease and pectase in healthy tobacco leaves has been described in a previous paper (Holden & Tracey, 1948). The plants used in getting the data given in this paper were a precisely similar series, but were infected with tobacco-mosaic virus.

Bawden (1943) and Wynd (1943) have reviewed the effects of virus infection on the metabolism of plants, and references to earlier work on tobacco infected with tobacco-mosaic virus can be found in their papers. The analyses described were made on tobacco plants supplied by Mr F. C. Bawden and Mr B. Kassanis, who were studying the effects of various fertilizer treatments on the susceptibility to infection and the multiplication of tobacco-mosaic virus. The data presented are restricted to a comparison of the nitrogen, phosphorus, protease and pectase levels in infected and healthy plants with different fertilizer treatments and the response to these treatments.

METHODS

Plants used. Tobacco (*Nicotiana tabacum* var. White Burley) plants were grown in pots in a heated glasshouse. The fertilizer treatments and levels were as described in the previous paper (Holden & Tracey, 1948).

Infection of the plants with tobacco-mosaic virus. In three experiments the plants were infected by rubbing five leaves with a virus preparation. These plants were harvested after 10–14 days, by which time only local virus multiplication had occurred. In the other two experiments the plants were infected when much younger, and grown for a period sufficient for the virus to spread systemically throughout the plant. The cultural history of the plants is given in Table 1.

Fractionation and analyses. The preparation of leaf fractions and their analysis has been described in the previous paper. pH determinations were made on spun sap using a glass electrode. Estimations of virus concentration were made on spun sap and washed fibre by Bawden & Kassanis (unpublished).

Analysis of data. The initial stages of the statistical analysis of the data were as described previously, except that the results from local and systemic infection were kept separate. The results for healthy controls were also analyzed, after these had been separated into groups corresponding to controls for local and systemic infection to eliminate seasonal differences. Standard errors for the means, and for the differences between means of healthy and infected plants, were calculated for both groups (systemic and local with their corresponding controls). A pooled standard error for the difference between fertilizer effects is given, as the individual standard errors were very similar.

Table 1. *Cultural history of the tobacco plants*

Date potted	Date of infection	Length of infection (days)	Average weight of plant* with full fertilizer supplement (g.)	Method of sampling (leaves taken)
11. x. 46	17. xii. 46	14	*I* 33·3 *H* 34·1	5
31. x. 45	12. ii. 46	10	*I* 106·0 *H* 92·0	5
4. ii. 46	17. iv. 46	10	*I* 52·6 *H* 105·2	5
21. v. 46	11. vi. 46	30	*I* 112·2 *H* 144·5	All
31. vii. 46	20. viii. 46	35	*I* 72·4 *H* 88·0	All

* Here and in later tables, *I* = infected; *H* = healthy.

RESULTS

The results obtained are given in Tables 2–6. In Table 3 relating to N, the N contents have been given both in terms of the total N, and also total N with virus N subtracted for the systemic experiments. Virus N figures were calculated from estimated amounts of virus on the assumption that its N content was 16·5 %.

The effects of systemic infection are summarized in Table 7.

The sap pH values are not recorded as there were no consistent differences either with different fertilizer treatments or with virus infection.

Table 2. *Dry matter content of tobacco plants*

Character determined	Condition of plant*	Extreme range	Mean	Standard error of difference between means of *I* and *H*†	Mean of untreated group	Mean of N, P and K group	Fertilizer effects N	P	K	N and P	Standard error of difference between fertilizer effects on *I* and *H*†
Wet weight of plants (g.)	*L*	22–424	137	±16·1	75	285	+ 30	+150	+14	+ 55	±32·3
	H	35–526	162		75	355	+ 54	+162	+40	+ 51	
	S	20–561	192	±19·6	78	462	+137	+188	+46	+109	±39·4
	H	62–722	269		136	581	+164	+220	+26	+133	
Wet weight of leaves (g.)	*L*	14–262	78	± 7·4	51	148	+ 18	+ 64	+ 4	+ 29	±14·7
	H	22–268	79		51	147	+ 21	+ 57	+14	+ 22	
	S	17–412	156	± 8·9	70	348	+ 99	+134	+33	+ 72	±16·4
	H	56–451	208		120	386	+101	+124	+14	+ 73	
Dry matter (% of wet weight)	*L*	7·3–16·9	11·5	± 0·35	11·1	11·9	− 0·65	+ 0·70	+0·22	− 0·05	± 0·71
	H	7·1–16·8	11·1		10·6	11·8	− 0·57	+ 0·77	+0·43	+ 0·25	
	S	9·6–13·9	11·7	± 0·42	10·9	11·6	− 0·31	+ 1·59	−0·11	− 0·59	± 0·85
	H	10·0–16·1	13·1		13·3	12·8	− 1·43	+ 2·13	−0·48	+ 0·13	
Total dry matter (g.)	*L*	1·5–43·9	9·8	± 1·81	5·9	20·6	+ 2·8	+ 8·9	+0·9	+ 4·4	± 3·63
	H	1·8–44·9	9·8		5·6	19·9	+ 3·2	+ 8·0	+2·1	+ 3·3	
	S	1·8–51·9	18·5	± 2·21	8·0	40·4	+11·4	+17·3	+3·3	+ 8·3	± 4·42
	H	6·3–64·5	28·3		16·9	49·7	+11·1	+20·1	±0	+10·3	
Fibre dry matter (% total dry matter)	*L*	40–60	48·5	± 0·93	49	49	− 1·5	+ 2·0	−0·5	− 1·0	± 1·87
	H	42–57	49·5		47	48	− 3·0	+ 3·5	−1·0	± 0	
	S	45–66	55·3	± 1·13	50	54	− 5·0	+ 9·0	+1·0	− 2·0	± 2·26
	H	45–65	53·0		54	56	− 4·0	+ 8·0	−1·0	± 0	

* Here and in later tables, *L* = locally infected; *S* = systemically infected.
† See Table 1, footnote.

Table 3. *Nitrogen content of tobacco plants*

Character measured	Condition of plant*	Extreme range	Mean	Standard error of difference between means of *I* and *H*†	Mean of untreated group	Mean of N, P and K group	Fertilizer effects N	P	K	N and P	Standard error of difference between fertilizer effects on *I* and *H*†
N (% of dry matter)	*L*	1·10–5·80	3·32	±0·16	2·88	3·47	+1·84	− 0·49	−0·36	+0·05	±0·31
	H	1·03–5·65	3·29		3·06	3·43	+1·61	− 0·53	−0·50	+0·12	
	S	2·29–6·20	4·19	±0·20	3·85	4·74	+2·02	− 1·06	−0·13	+0·18	±0·40
	H	1·27–6·89	3·19		2·52	2·91	+2·33	− 1·57	−0·14	−0·72	
Total N (mg.)	*L*	83–763	259	±59	158	499	+172	+182	− 27	+132	±117
	H	75–691	258		161	447	+171	+120	±0	+ 87	
	S	111–2050	765	±72	290	1823	+792	+619	+120	+477	±144
	H	231–3282	805		370	984	+807	+327	−113	+282	
Sap N (% of dry matter)	*L*	2·10–8·80	4·53	±0·21	3·64	4·70	+2·52	− 0·76	−0·31	−0·40	±0·41
	H	2·17–6·90	4·49		3·90	4·70	+2·41	− 1·26	−0·25	−0·50	
	S	3·15–8·95	5·30	±0·25	4·52	4·80	+2·43	− 1·54	−0·58	−0·71	±0·50
	H	1·84–9·23	4·28		3·13	3·61	+3·19	− 2·12	−0·41	−1·45	
Sap N (mg./ml.)	*L*	1·18–3·91	2·20	±0·11	1·83	2·36	+1·22	− 0·52	−0·13	−0·26	±0·23
	H	1·04–4·47	2·16		1·93	2·37	+1·15	− 0·62	−0·22	−0·14	
	S	1·48–4·24	2·82	±0·14	2·34	2·79	+1·62	− 0·79	−0·21	−0·11	±0·28
	H	1·16–4·52	2·27		1·72	2·01	+1·82	− 1·10	−0·24	−0·63	
Sap protein N (mg./ml.)	*L*	0·71–2·34	1·37	±0·08	1·24	1·75	+0·52	+ 0·03	−0·01	+0·13	±0·16
	H	0·61–2·62	1·28		1·16	1·74	+0·54	+ 0·04	−0·02	+0·18	
	S	1·11–2·80	1·84		1·57	2·20	+0·89	± 0	−0·10	+0·31	
	S	(0·57–2·26	1·30	±0·10	1·10	1·46	+0·81	− 0·26	−0·05	+0·16)	±0·20
	H	0·47–1·78	1·08		0·87	1·29	+0·77	− 0·17	−0·08	+0·14	
Sap sediment N (% of non-fibre N)	*L*	10·4–22·2	16·3	±1·1	16·9	16·4	−0·1	− 0·1	−1·1	+2·4	±2·3
	H	10·0–24·3	16·6		14·7	15·1	−1·4	+ 1·7	−1·1	+1·3	
	S	12·4–23·0	16·8	±1·4	18·8	13·9	−2·7	− 1·7	−1·2	+0·7	±2·8
	H	9·9–30·3	19·9		20·3	15·4	−5·4	− 1·7	+0·4	+0·6	
Fibre N (% of dry matter)	*L*	0·75–4·98	2·78	±0·15	2·64	2·92	+1·23	− 0·18	−0·47	+0·23	±0·31
	H	0·74–5·08	2·67		2·54	2·79	+1·07	− 0·07	−0·53	+0·37	
	S	2·07–4·90	3·53		3·51	3·86	+1·40	− 0·69	−0·16	+0·47	
	S	(0·51–3·77	2·41	±0·19	2·69	2·11	+1·14	− 1·37	−0·10	+0·19)	±0·37
	H	1·07–5·65	2·62		2·22	2·76	+1·77	− 0·82	−0·06	−0·14	
Fibre N (% total N)	*L*	34–48	41·2	±1·26	45	41	−5·1	+ 4·3	−3·4	+2·4	±2·5
	H	27–50	38·8		39	39	−2·8	+ 6·2	−1·8	+2·2	
	S	35–57	45·6	±1·56	44	56	−1·1	+10·3	+0·1	+6·4	±3·1
	H	29–59	47·2		48	54	−5·4	+13·9	+0·4	+5·1	

* See Table 2, footnote.

† See Table 1, footnote.

DISCUSSION

The data given in tabular form offer opportunities for extended discussion and speculation. It is proposed, however, to restrict the scope of the discussion, but increase its validity, by considering only differences in means of values for infected and healthy plants, and by excluding any that are not at least twice as great as the calculated standard error for differences between means.

Local multiplication of the virus for a period of about 10 days results in no significant change in the response of the plants to fertilizer supplements, and to a difference in composition between healthy and infected plants only in the percentage of total P that is in fibre.

Continued multiplication of the virus with its spread throughout the leaves has some effect on the response to fertilizer treatment, and causes profound changes in the size and composition of the leaves. There are four instances of significant changes in fertilizer response. The positive effect of P supplements on the total N of the leaves is increased and the K effect is reversed. The negative effect of N and the positive effect of P on the protease units/g. protein N of sap are both significantly enhanced. In

Table 4. *Phosphorus content of tobacco plants*

Character measured	Condition of plant*	Extreme range	Mean	Standard error of difference between means of *I* and *H*†	Mean of untreated group	Mean of N, P and K group	Fertilizer effects N	P	K	N and P	Standard error of difference between fertilizer effects on *I* and *H*†
P (% of dry matter)	*L*	0·096–0·593	0·256	±0·020	0·131	0·244	+ 0·016	+ 0·175	−0·032	− 0·022	±0·040
	H	0·092–0·455	0·238		0·198	0·472	− 0·003	+ 0·216	−0·021	− 0·008	
	S	0·182–0·600	0·318	±0·024	0·214	0·428	+ 0·030	+ 0·210	−0·080	− 0·046	±0·048
	H	0·142–0·470	0·247		0·155	0·330	+ 0·030	+ 0·170	−0·040	− 0·016	
Total P (mg.)	*L*	5·32–66·35	25·20	±2·1	5·51	35·84	+ 7·73	+31·03	−2·20	+ 8·21	±4·3
	H	2·51–36·46	24·20		6·70	43·81	+ 3·84	+28·80	+2·94	+ 4·06	
	S	4·30–165·4	65·50	±2·7	17·00	158·60	+50·84	+90·11	+1·64	+46·75	±5·4
	H	10·10–187·2	75·40		25·26	157·00	+42·24	+95·01	−9·05	+38·95	
Sap P (% of dry matter)	*L*	0·10–0·59	0·357	±0·054	0·100	0·230	− 0·043	+ 0·236	−0·010	− 0·070	±0·108
	H	0·09–0·78	0·295		0·130	0·430	− 0·038	+ 0·330	−0·033	− 0·034	
	S	0·16–0·89	0·432	±0·066	0·220	0·550	− 0·070	+ 0·430	−0·050	− 0·050	±0·133
	H	0·14–0·72	0·448		0·180	0·540	+ 0·066	+ 0·529	−0·189	+ 0·054	
Fibre P (% of dry matter)	*L*	0·080–0·380	0·200	±0·013	0·130	0·221	+ 0·040	+ 0·100	−0·050	− 0·010	±0·026
	H	0·080–0·400	0·183		0·120	0·220	+ 0·029	+ 0·128	−0·046	+ 0·018	
	S	0·185–0·395	0·250	±0·016	0·200	0·322	+ 0·061	+ 0·070	−0·018	+ 0·061	±0·031
	H	0·140–0·390	0·198		0·160	0·241	+ 0·051	+ 0·089	−0·039	− 0·039	
Fibre P (% total P)	*L*	32–56	42·5	±1·4	45·0	39·5	+ 0·3	− 0·6	+0·4	− 0·3	±2·8
	H	26–55	39·6		43·6	36·3	+ 1·6	− 4·6	−0·9	+ 6·1	
	S	27–56	44·3	±1·8	47·0	40·5	+ 3·6	−10·0	−1·1	+ 2·0	±3·5
	H	29–63	45·9		54·5	43·0	+ 4·0	− 7·8	−5·5	+ 3·3	

* See Table 2, footnote.

† See Table 1, footnote.

general, however, it may be said that the response to fertilizers of infected plants is similar to that shown by healthy plants.

Infection in the early stages of growth, leading to systemic spread of virus is well known to result in a yellowing of the leaves and a stunting of the plant. This stunting is shown in Table 2 as a reduction in the wet weight of the leaves, and there is a greater proportionate reduction of total dry matter, resulting in a wetter leaf. The decrease in dry matter is accompanied by a decrease in the total P, but the decrease in P content is less than that of dry matter so there is an increase in the P as percentage of total dry matter, and as percentage of dry matter of the fibre fraction. The increase is greater than can be accounted for by virus P.

During the yellowing of the leaves in tobacco-mosaic infection there may be a reduction in the chlorophyll content by as much as 60 % (Peterson & McKinney, 1938). The reduction in chlorophyll is presumably accompanied by a decrease in the amount of protein with which it is associated. Woods & Du Buy (1941) have shown that, particularly under conditions of N starvation, chromoprotein diminishes in leaves as the amount of virus increases. In the present experiments there was a significant decrease in infected leaves in the sap-sediment N fraction, which contains much of the chromoprotein. As the leaves taken from infected plants were in various physiological states, from small dark green and large yellow green to completely yellow leaves, the difference is not as great as would be expected if yellow leaves only from infected plants were compared with leaves of the same age from healthy plants.

The reduction in the carbohydrate content of tobacco leaves infected with tobacco-mosaic virus found by Dunlap (1930, 1931) agrees with the findings reported here, for, while there is a decrease in the total dry matter, there is no decrease in total N. Dunlap found that there was a greater decrease in the starch and sugar content than in that of the other carbohydrates. This would be in agreement with the present findings that there was a greater loss of soluble fractions, which resulted in a greater proportion of the dry matter being in the fibre. Cordingley, Grainger, Pearsall & Wright (1934) also found a decrease in total carbohydrate, but found that the proportion of the various fractions was the same in infected and healthy plants. These different results may be due to the different methods of fractionation and estimation used.

As systemic infection has no significant effect on the total N of the leaves it leads to an increase in N as percentage of dry matter. The distribution of N between fibre and soluble fractions is not altered,

Table 5. *Protease content of tobacco plants*

Character measured	Condition of plant*	Extreme range	Mean	Standard error of difference between means of *I* and *H*†	Mean of untreated group	Mean of N, P and K group	Fertilizer effects N	P	K	N and P	Standard error of difference between fertilizer effects on *I* and *H*†
Total protease (units/g. dry weight)	*L*	1·4–10·5	4·2	± 0·34	3·7	5·0	−0·7	+1·3	−0·2	−0·4	± 0·68
	H	2·0– 8·9	4·2		3·3	4·9	−0·5	+1·4	+0·4	+0·1	
	S	1·4–7·1	3·3	± 0·42	2·6	4·5	+0·4	+1·5	+0·2	+0·6	± 0·85
	H	0·6–4·1	2·7		2·2	3·8	±0·0	+0·7	+0·3	+0·6	
Protease (units/g. protein N)	*L*	48–545	186	±20	185	148	−70	+18	−49	−58	±40
	H	35–428	183		136	184	−91	+74	+24	−26	
	S	36–225	101	±24	110	154	−43	+56	+15	−18	±48
	H	20–203	117		78	100	−55	+41	±0·0	+25	
Protease (units/ml. sap)	*L*	0·04–0·60	0·34	± 0·031	0·29	0·46	−0·13	+0·15	−0·01	+0·04	± 0·062
	H	0·07–0·63	0·34		0·25	0·39	−0·13	+0·08	+0·08	−0·04	
	S	0·07–0·42	0·22	± 0·038	0·17	0·26	−0·01	+0·10	+0·02	+0·01	± 0·077
	H	0·03–0·49	0·25		0·19	0·33	−0·05	+0·12	+0·03	+0·02	
Protease (units/g. dry weight sap)	*L*	1·0–15·0	7·1	± 0·62	5·6	9·2	−2·6	+3·4	−0·4	+0·5	± 1·25
	H	1·5–13·3	7·1		5·0	7·5	−3·3	+1·4	+2·4	−1·3	
	S	1·2– 8·8	4·3	± 0·76	6·7	3·0	−0·9	+2·0	+0·4	−0·5	± 1·53
	H	0·5–10·5	4·9		3·7	0·7	−1·5	+2·4	+0·5	−0·1	
Protease (units/g. protein N of sap)	*L*	36–792	290	±39·9	236	293	−230	+154	−16	− 37	±80·0
	H	38–892	316		213	251	−248	+140	+90	−125	
	S	27–350	141	±48·6	112	127	−78	+ 67	+23	− 41	±97·2
	H	25–1040	300		204	261	−279	+224	+78	−126	
Protease (units/g. dry weight of fibre)	*L*	1·0–11·7	3·7	± 0·38	3·4	5·1	+0·4	+0·7	+0·6	+0·6	± 0·77
	H	1·4–12·4	3·4		2·7	4·9	+0·9	+1·7	−0·3	+0·7	
	S	1·7–8·8	3·4	± 0·47	2·5	4·2	+1·0	+1·3	−0·7	+0·9	± 0·94
	H	1·2–3·8	2·4		1·8	3·6	+0·6	+0·2	+0·4	+0·7	
Fibre (units/g. N)	*L*	21–284	128	± 8·6	121	149	−44	+37	+27	− 6	±17·3
	H	22–262	132		107	159	−34	+73	+ 9	−12	
	S	35–196	93	±10·6	68	105	−16	+55	− 9	+ 1	±21·2
	H	26–150	98		85	126	−43	+33	+23	+20	

* See Table 2, footnote. † See Table 1, footnote.

Table 6. *Pectase content of tobacco plants*

Character measured	Condition of plant*	Extreme range	Mean	Standard error of difference between means of *I* and *H*†	Mean of untreated group	Mean of N, P and K group	Fertilizer effects N	P	K	N and P	Standard error of difference between fertilizer effects on *I* and *H*†
Fibre (units‡/g. dry matter)	*L*	0·10–0·61	0·29	±0·050	0·35	0·24	+0·125	− 0·234	−0·013	−0·026	±0·10
	H	0·07–0·51	0·26		0·34	0·20	+0·106	− 0·216	−0·009	−0·028	
	S	0·08–0·95	0·46		0·55	0·41	+0·194	− 0·340	+0·081	−0·038	
	H	0·08–1·31	0·42		0·46	0·25	+0·225	− 0·482	±0	−0·103	
Fibre (units/g. N)	*L*	4·35–22·20	11·60	±1·50	15·30	11·28	−1·37	− 6·50	+2·16	+2·90	±3·00
	H	4·45–17·20	9·81		13·01	9·07	−0·16	− 5·32	+1·68	+0·72	
	S	3·78–24·10	12·33		15·50	10·61	+1·62	− 7·37	−0·72	+2·16	
	H	6·70–33·45	15·58		21·92	7·65	−2·25	−14·65	+0·69	+0·69	

* See Table 2, footnote. † See Table 1, footnote.

‡ For definition of units see Holden (1946).

and there is a corresponding increase in N as percentage of dry matter of both sap and fibre fractions. One of the results of infection is an increase in the rate at which the leaves yellow, which leaves of healthy plants do with increased age. The yellow healthy leaves, like the infected leaves, have a higher water content than healthy green leaves (Smirnow, 1940), but, unlike infected leaves, have less N/100 g. dry matter. If virus N is subtracted from the total N the infected leaves still do not have less N/100 g. dry matter, so infection cannot be regarded simply as a premature ageing of the leaves.

Table 7. *Effects of systemic infection of tobacco plants*

Decrease in:	Wet weight of plants
	Wet weight of leaves
	Total dry matter
	Total phosphorus
	Dry matter as % wet weight
	Sap sediment N as % non-fibre N
	Protease/g. protein N of sap
	Pectase/g. N of fibre
Increase in:	Total N as % dry matter
	Fibre N as % dry matter
	Sap N as % dry matter
	Total P as % dry matter
	Fibre P as % dry matter
	Sap N/ml.
	Protein N/ml.
	% total dry matter as fibre
	Total protease/g. dry weight
	Fibre protease/g. dry weight
No significant change in:	Total N
	% total N on fibre
	% total P on fibre
	Sap P as % dry matter
	Total units protease/g. total protein N
	Fibre protease/g. N
	Pectase/g. dry matter of fibre

Considering the mean of all treatments, about one third of the total N is virus N, in agreement with Bawden & Pirie (1946). But the range is from 10 to 65 % of the total N as virus N, and in one experiment (with plants given P but no N) 80 % of the fibre N was virus N. It is certain that the protein components of systemically infected leaves are affected differently, as, for example, the decrease in chromoprotein mentioned earlier. Several authors have referred to changes in enzyme levels caused by virus-infection (Balls & Martin, 1938; Peterson & McKinney, 1938; Wynd, 1942). In the present experiments systemic infection affected protease and pectase levels differently. Fibre pectase was related to non-virus N, while fibre protease was related to total N levels. Sap protease, on the other hand, was related to non-virus protein rather than to total protein N.

SUMMARY

1. The effects of supplements of nitrogen and phosphorus on the nitrogen and phosphorus content of leaf fractions of tobacco plants with local and systemic multiplication of tobacco-mosaic virus were similar to those found with healthy plants.

2. The effect of local multiplication of tobacco-mosaic virus on the composition of the leaf fractions studied was negligible.

3. The effect of systemic multiplication of tobacco-mosaic virus on the composition of the fractions studied was profound.

4. Total nitrogen was not affected, while dry matter and phosphorus were reduced.

5. The levels of the two enzymes studied were affected differently. Fibre protease was related to total fibre nitrogen while pectase appeared to be related to non-virus fibre nitrogen.

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Glycerylphosphorylcholine and Choline Glycerophosphate

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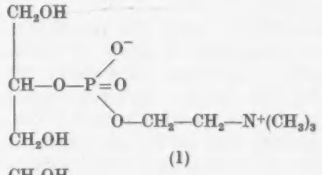
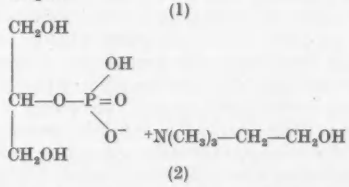
In 1938–9 at the British Postgraduate Medical School, King & Aloisi isolated from dried pancreas a glycerophosphoric ester of choline which was found to be readily hydrolyzable. Evidence was also obtained for the existence of a dicholine ester. This work was published only in 1945 owing to war difficulties. In the same year Schmidt, Hershman & Thannhauser (1945) reported the isolation of α-glycerylphosphorylcholine from the same material. They also found that the ester was very rapidly split by N-hydrochloric acid.

We have now tried to synthesize the choline esters of both α- and β-glycerophosphoric acid in order to examine their metabolic behaviour in animal tissues and other cells. The products obtained by the few methods recorded in the literature for the synthesis of β-glycerylphosphorylcholine (Arnold, 1940; Ravazzoni & Fenaroli, 1940; Belfanti, Ercoli & Francioli, 1940; Mihalik, Jeney & Uri, 1941) seemed unlikely to us to be esters of choline (1), but rather to be salts of choline and β-glycerophosphoric acid (i.e. β-glycerophosphate of choline) (2).



(1)



(2)

After having attempted the synthesis in several ways which are briefly described, we decided to follow a method analogous to that used by Kabashima (1938) for the synthesis of lecithins (though this still requires assessment). Ravazzoni & Fenaroli (1940) also attempted this, but with no clear result. Bromocholine picrate and silver glycerophosphate were treated in hot absolute ethanol for several hours, followed by removal of picric acid and purification.

EXPERIMENTAL

Preparation of choline β-glycerophosphate

Method of Arnold (1940). This consists in boiling bromocholine bromide (trimethyl-β-bromoethylammonium bromide) and silver β-glycerophosphate in absolute ethanol. Arnold obtained a hygroscopic crystalline product, m.p. 104–105°, with a P/N atomic ratio = 1 and good analytical figures; it was precipitated from ethanolic solutions by ether and other solvents. In Arnold's paper there is no report about either a test for free choline (Florence's reaction, cf. Stanek, 1908) or the behaviour of the substance on hydrolysis. But he quoted a property of his substance the significance of which could hardly be misleading; the cadmium precipitate of his 'ester' did not contain nitrogen. These properties were sufficient to raise the question whether Arnold's product was really an ester of choline.

In our experiments we boiled equivalent amounts of silver β-glycerophosphate (from the Na salt; 100% pure β-isomer by HIO_4 analysis) and bromocholine bromide (Krüger & Bergell, 1903; m.p. 230°) in absolute ethanol for 7 hr. in a N_2 atmosphere with thorough stirring. After removal of AgBr and several precipitations with ether, we obtained from ethanol-ether mixture a fairly good crystalline product, which showed the same properties as described by Arnold. It was very hygroscopic, with m.p. 104° when thoroughly dried over P_2O_5. (Found: N, 5·15; P, 11·24; choline (method of Roman, 1930), 40·3. Calc. for $C_8H_{22}O_7NP$: N, 5·09; P, 11·27; choline, 44·0%.) We obtained good crystals also from ethanol-acetone and ethanol-pyridine mixtures (see Pl. 2, Fig. 1).

Evidence that the substance is a choline salt of β-glycerophosphoric acid. (*a*) Ammonium reineckate gave from aqueous solutions crystals like choline reineckate which did not contain P. (*b*) The Florence test was positive and choline was estimated quantitatively by the Roman (1930) method. (*c*) With $Ba(OH)_2$ the product yielded a precipitate which contained P but no N (Ba β-glycerophosphate). (*d*) With NH_4OH in absolute ethanol at 2–4° crystals of ammonium β-glycerophosphate were formed. (*e*) Basic lead acetate, with and without addition of NH_4OH, precipitated Pb glycerophosphate when the aqueous solution of the substance was sufficiently concentrated (4–5%). Weaker solutions gave a partial precipitate (17% from a 0·64% solution) of a mixture with P/N = 1. (*f*) The pH titration curve showed a pK of 6·36 which corresponded to that of β-glycerophosphoric acid.

Preparation of choline α-glycerophosphate

This was not prepared by Arnold, but we followed the same procedure as for the β-isomer. The Na_2 α-glycerophosphate from which we started contained 88 % of pure α-isomer (HIO_4 analysis). The yield was practically a quantitative one. The product was a light yellow hygroscopic syrup soluble in ethanol, insoluble in ether. We were not able to obtain crystals from dry ethanol-ether mixtures either after 3 months' keeping at 0°, or after 24 hr. over solid CO_2; under these conditions the β-isomer undergoes massive crystallization.

In the first steps of purification from ethanol solutions small white precipitates were formed and discarded. Finally the syrup became more and more difficult to dissolve in anhydrous cold ethanol. The P/N ratio by weight was 2·3 (theory, 2·21) after several precipitations; this showed that the substance was a well-defined compound. The evidence for a salt-like nature was the same as described for the β-isomer. The pH titration curve was the same as for α-glycerophosphoric acid over the range studied.

Method of Mihalik et al. (1941). We did not see the original paper which was published in the Proceedings of the Hungarian Physiological Society. The method was reported to consist in putting together equivalent amounts of choline base and β-glycerophosphoric acid (*apparently in aqueous solution*), and boiling them for 36 hr. In the report it is stated that the product (m.p. 104–105°) did not contain free choline as the Florence test was negative (cf. Stanek, 1908). This is a rather doubtful result, since the melting point is the same as that of Arnold's (1940) product which, as we showed above, is actually a salt. Furthermore it is impossible to understand how an ester could be yielded by the mere contact of β-glycerophosphoric acid and choline in water; also in ethanol the affinity of the secondary OH group of glycerophosphoric acid for the N would be overwhelming.

We have tried to obtain the choline salt of β-glycerophosphoric acid by mixing equivalent amounts of β-glycerophosphoric acid and choline base (from choline chloride and wet Ag_2O, or from trimethylamine and ethylene oxide) in water; the mixture was dried at room temperature *in vacuo* over P_2O_5, dissolved in absolute ethanol and precipitated several times with dry ether. From the ethanolic solutions of the syrupy precipitate we got beautiful crystals by adding dry ether or pyridine. The crystals, dried *in vacuo* over P_2O_5 at 70–75° for 30 hr., were still extremely hygroscopic and showed a m.p. 93–94°. They gave the following analytical figures. Found: N, 4·26; P, 9·96; P/N, 2·3. Calc. for $C_8H_{22}O_7NP.H_2O$: N, 4·50; P, 9·96 %; P/N, 2·2.

Inorganic P and N were absent and all the N was present as choline. Attempts to eliminate the water of crystallization were unsuccessful (over P_2O_5 at 75° *in vacuo* for 30 hr.). Apart from the above-mentioned difference, the chemical behaviour of the substance was identical with that of Arnold's product, and therefore gave further evidence of the salt-like nature of the latter.

Methods of Ravazzoni & Fenaroli (1940). These authors gave two methods for the synthesis of the choline ester of β-glycerophosphoric acid. One is the same as that of Arnold, with the irrational variant of operating in water as well as in methanol. The other is based on the reaction of bromocholine picrate with monosilver glycerophosphate suspended in water. After removal of picric acid and purification, the authors obtained a crystalline product which showed good analytical figures for $C_8H_{22}O_7NP$. They did not give the melting point of the substance, but they stated that the Florence test was negative and became positive only after hydrolysis with hot HCl. No information was given about the yield.

We repeated the preparation as described by Ravazzoni & Fenaroli and did not get a crystalline product, but only a little light yellow syrup with a very low N content (P/N ratio by weight = 5), which with basic lead acetate gave a precipitate free from N. The Florence test was frankly positive, while in dilute solutions we had no evidence of choline reineckate crystals. After hydrolysis with 2N-HCl at 100° the same dilute solution showed crystals of reineckate; it is well known that ammonium reineckate gives silky crystals with trimethylamine, which is, however, negative to the Florence test.

Conclusion. It is obvious that none of these methods is likely to give the choline esters of glycerophosphoric acid. Actually a crystalline compound similar to that described by Arnold can be obtained simply by mixing equivalent amounts of β-glycerophosphoric acid and choline base in aqueous solution.

α- and β-Glycerylphosphorylcholine

Being aware from our previous work that the chief difficulty in preparing the esters of choline with glycerophosphoric acid lies in the strong affinity of the secondary OH group of glycerophosphoric acid for the N of choline, we tried at first to obtain the esters by starting with glycerophosphoric acid and ethylenimine in anhydrous media, following the model proposed by Christensen (1940) for the synthesis of phosphorylcolamine. We intended to attempt the methylation of the glycerylphosphorylcolamine eventually formed, according to the method of Trier (1912). The ethylenimine was prepared from colamine (obtained according to Knorr, 1897) following the method of Wenker (1935). But our attempt was completely unsuccessful, as we got a white waxy substance with the expected P/N ratio, but which was again a colamine salt of glycerophosphoric acid and not the expected ester. Actually the precipitate with basic lead acetate did not contain N. It was significant in this connexion that, on the contrary, phosphorylcolamine was easily obtained by this method (and, even with better yields, by the direct reaction between phosphoric acid and colamine at high temperature). These

results convinced us that it was very difficult to prevent salt formation between the secondary OH group of glycerophosphoric acid and the amino group of colamine, or choline, and that apparently this secondary OH group is far more difficult to esterify than the primary one.

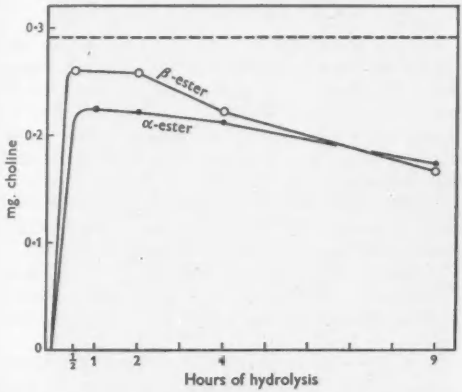


Fig. 2. Hydrolysis of synthetic α- and β-glycerylphosphorylcholine (0·55 mmol.) in 2N-HCl. The dotted line is at the theoretical value.

We then decided to try the picrate of bromocholine and silver glycerophosphate in anhydrous conditions. We assumed that the presence of the picric acid radical in the bromocholine molecule could prevent the salt linkage with the glycerophosphoric acid, which should be esterified owing to the Ag-Br affinity.

Preparations. We followed the same procedure for both α- and β-glycerylphosphorylcholine, using silver α- and β-glycerophosphates from the corresponding sodium salts (α-isomer 88%; β-isomer 100% pure with HIO_4) and bromocholine picrate (m.p. 160°) prepared from bromocholine bromide (m.p. 230°) and picric acid in ethanol.

Equimolecular amounts of Ag_2 glycerophosphate and bromocholine picrate were refluxed in absolute ethanol in an oil bath for 60 hr. in darkness. The clear ethanolic solution was filtered and evaporated to dryness *in vacuo*. The solid residue was extracted exhaustively with acetone. The dry substance was soluble in water, but no longer soluble in ethanol; it contained Ag. The latter was removed with H_2S from aqueous solution, which was filtered and brought to dryness again *in vacuo* at low temperature. Before complete dryness was reached we found it necessary to neutralize the syrup in order to prevent hydrolysis. The dry residue was a light-yellow sticky syrup, almost insoluble in cold absolute ethanol, but easily soluble in water-ethanol mixtures. For both α- and β-preparations the yields were extremely low, and as we had to utilize them for biological purposes we did not try to establish other properties than those summarized below.

The aqueous solutions were about pH 4; inorganic P and N were absent; P/N ratio = 2·22 (theoretical = 2·21); Florence and reineckate tests were negative; after hydro-

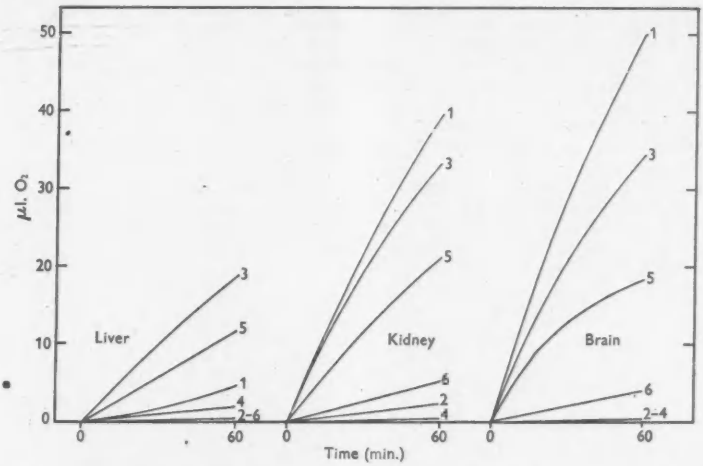


Fig. 3. Oxidation of different substrates by guinea pig liver, kidney and brain tissues. The curves show the extra oxygen uptake due to the added substrate. 1 = Na_2 α-glycerophosphate; 2 = Na_2 β-glycerophosphate; 3 = choline α-glycerophosphate; 4 = choline β-glycerophosphate; 5 = glyceryl-α-phosphorylcholine; 6 = glyceryl-β-phosphorylcholine. In every set of experiments, 0·5 ml. of 20% tissue suspensions, homogenized according to Potter & Elvehjem (1936); 1·5 ml. M/15-PO_4 buffer; 0·3 ml. of 0·06M-substrate solution (0·0182 mmol.) were used, with physiological saline to 3 ml. Temperature, 38°.

lysis with 2N-HCl at 100° considerable amounts of choline were set free during the first 15–30 min. (cf. Roman, 1930); these amounts in both α- and β-glycerylphosphorylcholine were practically consistent with the theoretical figures (see Fig. 2), bearing in mind that during the hydrolysis the choline values tend to drop owing to destruction of choline. These results are quite similar to those found by King & Aloisi (1945) and by Schmidt, Herschman & Thannhauser (1945) working with the natural α-glycerylphosphorylcholine extracted from beef pancreas. This gave assurance that with this method it is really possible to obtain the choline esters of glycerophosphoric acids. For our biological purposes the method was quite satisfactory for we had sufficient amounts of fairly pure esters to carry on our respirometric measurements; but from the chemical point of view the method is less satisfactory owing to the very low yields. With this method, however, it should be possible to obtain a dicholine ester by working with 1 mol. of silver glycerophosphate and 2 mol. of bromocholine picrate.

Biological behaviour. The oxidations of α- and β-glycerylphosphorylcholine, in comparison with sodium α- and β-glycerophosphate, and with choline α- and β-glycerophosphate, by living tissues *in vitro* are recorded by us in another paper (Aloisi & Buffa, 1947). We want here only to note (see Fig. 3) that in all cases (following Warburg procedure) the α-isomers are better utilized than the β-isomers, and that the choline esters and choline salts are oxidized by liver more actively than the sodium glycerophosphates, while other tissues (kidney, brain) oxidize the simple glycerophosphates better than the esters. In our conditions choline oxidase worked at a very slow rate. Other tests, with bacteria, are in progress.

SUMMARY

1. It is shown that the methods found in the literature for the synthesis of β-glycerylphosphorylcholine are likely to give only the choline salts of β-glycerophosphoric acid.

2. Choline α- and β-glycerophosphate (the latter in crystalline form) have been prepared and described.

3. The difficulty in obtaining the choline and colamine esters lay in the fact that ionic reaction preponderated and esterification was prevented.

4. Linkage between choline and glycerophosphoric acid was obtained by treating bromocholine picrate with silver α- and β-glycerophosphates under anhydrous conditions.

5. Evidence is given of the formation by this procedure of the choline esters, the α-isomer of which had the same properties as the naturally occurring compound.

6. Preliminary data on the biological behaviour of the esters in living tissues are briefly recorded.

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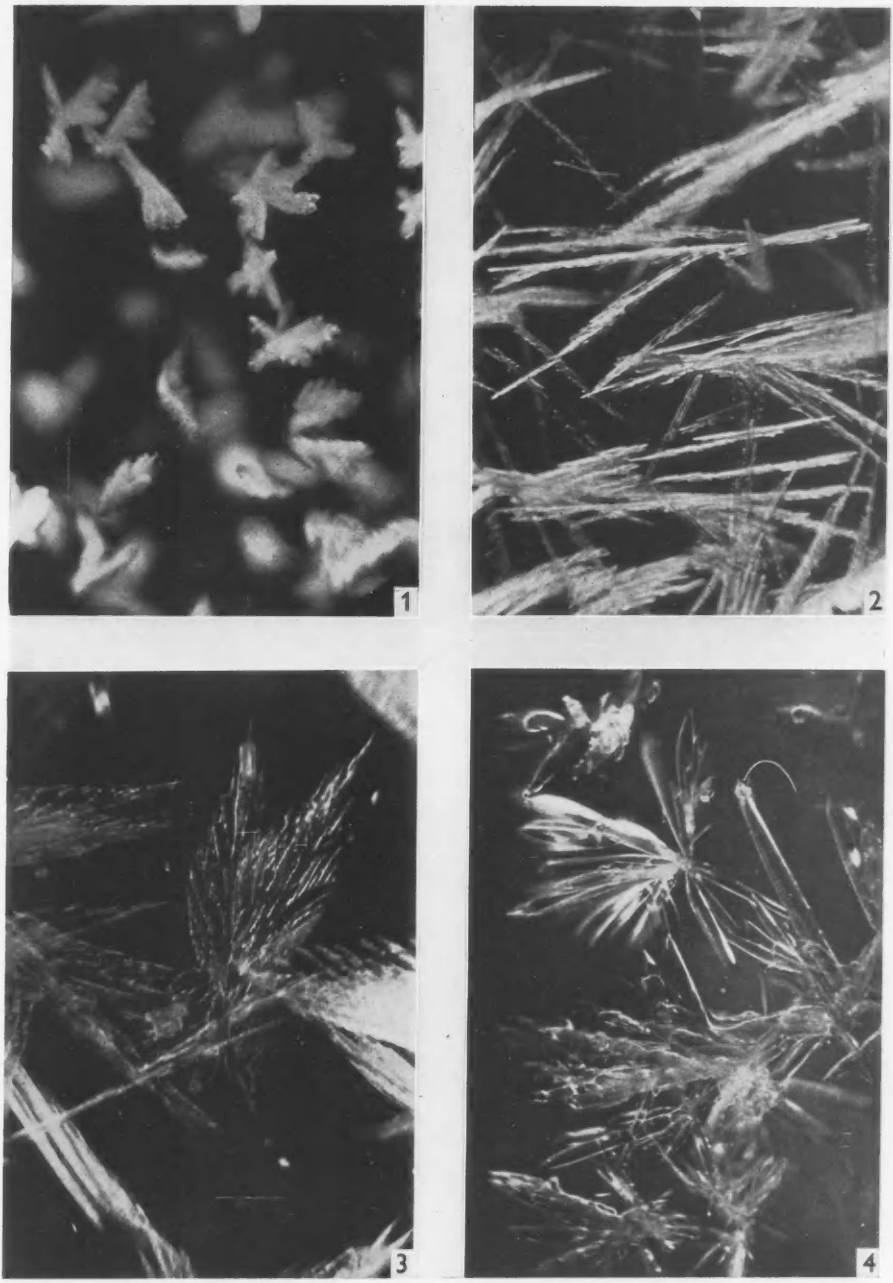

Fig. 1. Choline β-glycerophosphate. (1) Method of Arnold from ethanol-acetone. (2) The same from ethanol-pyridine. (3) The same from ethanol-ether. (4) Method by direct reaction of glycerophosphoric acid with choline, from ethanol-ether.